Expression and Function of the ATP Dependent Chromatin Remodeler Imitation Switch in *Xenopus laevis*

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EXPRESSION AND FUNCTION OF THE ATP DEPENDENT CHROMATIN REMODELER IMITATION SWITCH IN *XENOPUS LAEVIS*

A

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By

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Abstract

One of the first ATP-dependent chromatin remodeling complexes was first identified and characterized over ten years ago. Since then, the number of distinct ATP-dependent chromatin remodeling complexes and the variety of roles they play in nuclear processes have become dizzying (Flaus and Owen-Hughes 2003; Lusser and Kadonaga 2003; Martens and Winston 2003; Vaquero et al. 2003; Dirscherl 2004). Some of the processes include transcription, replication, repair, recombination, and sister chromatid cohesion. The SWI/SNF-related ATP-dependent remodelers are divided into a number of subfamilies, all related by the SWI2/SNF2 ATPase at their catalytic core. In nearly every species where researchers have looked for them, one or more members of each subfamily have been identified. Here I have investigated the ATP dependent chromatin remodeler ISWI. I have shown that *Xenopus* ISWI, which is in its own subfamily, has a critical function in developing neural tissue. Whole mount *in situ* hybridization shows *ISWI* localized in neural tissue including the eye and developing neural tube. Injection of antisense *ISWI* RNA, morpholino oligonucleotides or dominant-negative *ISWI* mutant mRNA into fertilized eggs misregulates genes involved in patterning and development, such as *BMP4* and *Sonic hedgehog (Shh)*, and ISWI binds to the *BMP4* gene in vivo. Partial inhibition of ISWI function results in aberrant eye development and the formation of cataracts. These data suggest a critical role for ISWI chromatin remodeling complexes in neural development.

Table of Contents

List of Figures

List of Tables

List of Other Materials

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List of Appendices

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Chapter 1

Introduction

The eukaryotic nucleus houses DNA, the genetic material of the organism. DNA is the blueprint for making thousands of different molecules in the body. In order for a functional product to be produced DNA must first be transcribed into RNA and then translated into protein (figure 1). The resulting proteins have many functions in the body, including structural and enzymatic roles.

The nucleus of a mammalian cell averages approximately 10 μm in diameter, however, it houses over 2 meters of DNA. This is possible through complex interactions that condense the DNA down into tight coils. The first step in compaction is the winding of the DNA around proteins called histones. This DNA-protein complex is called a nucleosome and is repeated over and over again. The nucleosome consists of 146 bp of DNA wrapped nearly two times around a core histone octamer that consists of two copies each of histone H2A, H2B, H3 and H4. The linker histone H1 interacts with the nucleosomal core as well as linker DNA. The nucleosome is the core repeating subunit of "chromatin", the term used to describe the entire protein-DNA network in the nucleus (figure 2). Negatively charged DNA and positively charged histones attract each other through electrostatic forces. The nucleosomes then wind around each other furthering the compaction into a 30 nm chromatin fiber (figure 3).

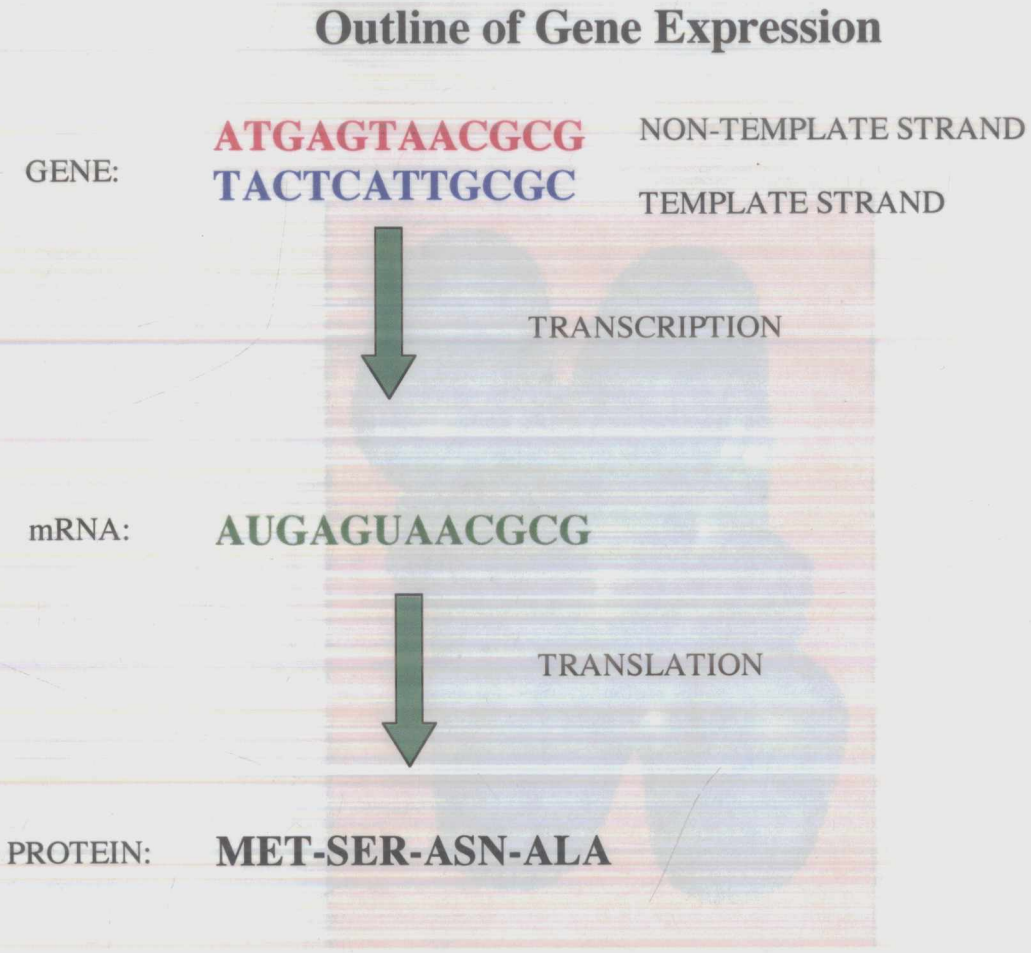

Figure 1.1 Central dogma. DNA is transcribed into RNA then translated into protein.

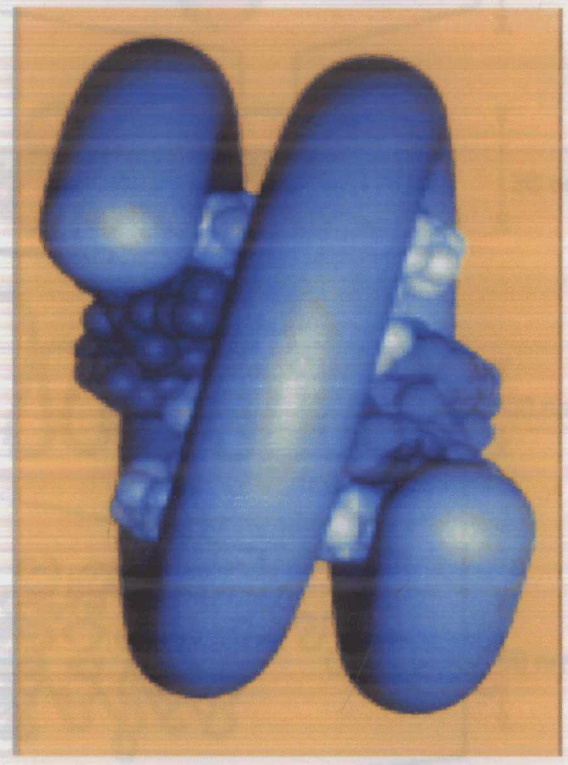

Figure 1.2 The nucleosome. 146 bp of DNA is wound around an octamer of histone proteins and is called a nucleosome (Arents and Moudrianakis 1993).

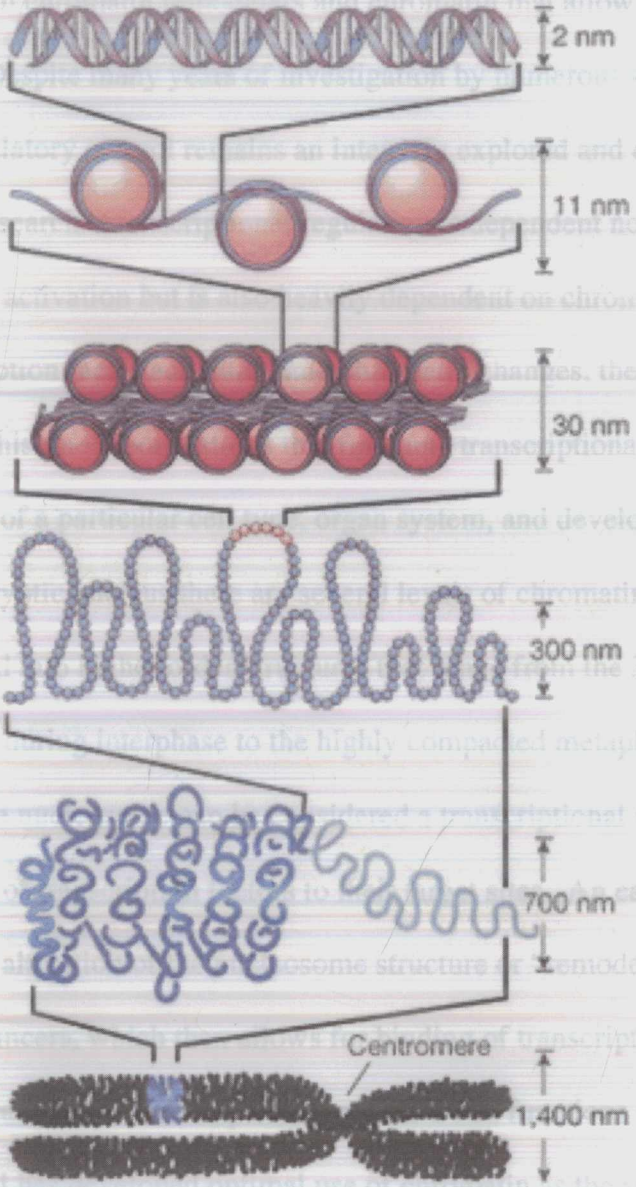

Figure 1.3 Condensation of DNA. DNA is wound around the octomer of core histones to from the nucleosome, which is then further condensed to fit inside the nucleus of a cell. (taken from Felsenfeld and Groudine Nature 2003)(Felsenfeld and Groudine 2003)

The question is that with all of this compaction, how is our DNA accessed by transcription factors and enzymes to be transcribed into RNA? It is the intricate interactions between chromatin remodelers and chromatin that allow DNA to be available for transcription. Despite many years of investigation by numerous scientists, transcriptional regulatory control remains an intensely explored and continuously evolving field of research. Transcriptional regulation is dependent not only on transcription factor activation but is also heavily dependent on chromatin structure. In addition to transcription factor activation and chromatin changes, there is an expanding array of additional histone modifications that fine tune transcriptional regulation for the specific conditions of a particular cell type, organ system, and developmental stage.

In the eukaryotic nucleus there are several levels of chromatin compaction. Arrays of nucleosomes fold into higher order structures that range from the 30 nm diameter chromatin filament during interphase to the highly compacted metaphase chromosomes during mitosis. The nucleosome can be considered a transcriptional repressor because it impedes the access of transcription factors to their target sites. An early step in gene activation involves alteration of the nucleosome structure or "remodeling" at active promoters and enhancers, which then allows for binding of transcription factors.

Every nuclear process that requires access to DNA functions in the context of chromatin. The cell has developed optimal use of chromatin as the substrate for DNA-directed processes. For instance, histones are subject to a number of covalent

modifications such as acetylation, phosphorylation, methylation and ubiquitination (figure 4). These modifications serve to alter the biochemical properties of chromatin as well as to provide signals that regulate the activities of other factors, known as the "histone code." A combination of chromatin remodeling enzymes and histone modifying enzymes establish a steady level of transcriptional control in all eukaryotes.

In addition to covalent histone modification, cells also use ATP-dependent chromatin remodeling as a means of controlling access to DNA. This remodeling is performed by multi-subunit complexes that use the energy of ATP hydrolysis to disrupt histone-DNA interactions (figure 5). In the next chapter we will discuss the rather large SWI2/SNF2 superfamily of ATP-dependent chromatin remodelers in detail.

Imitation Switch (ISWI) is a subfamily of the SWI2/SNF2 superfamily. ISWI is a common subunit of many different complexes. The ISWI subunit is present in at least 7 complexes in mammals, three in *Drosophila*, four in *Xenopus*, and four in budding yeast. One *ISWI* homolog has also been found in *Arabidopsis*. The extreme diversity of ISWI complexes within the same organism as well as homology between organisms suggests a wide range of essential functions. One example is ISWI's association with WSTF (Williams syndrome transcription factor) in the WICH complex. WSTF codes for a transcription factor which is deleted (along with several other genes) in the genetic disorder Williams-Buernes syndrome. This syndrome is characterized by severe developmental delays, unusual facial appearances and specific cognitive and personality

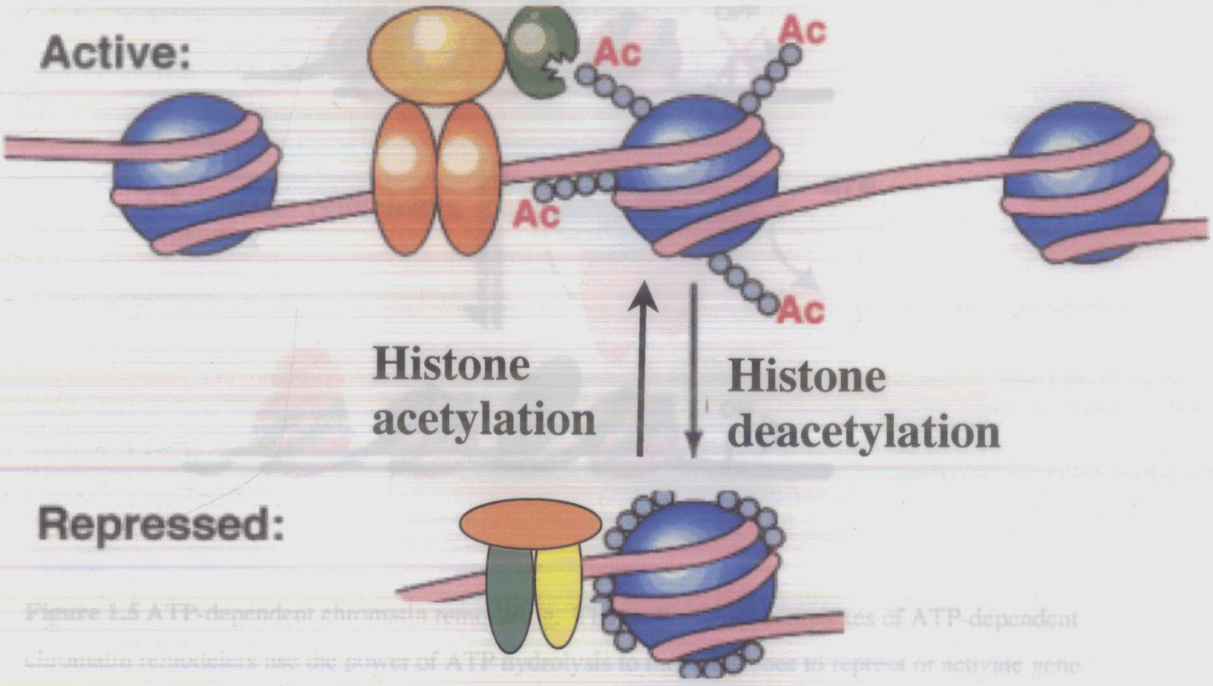

Figure 1.4 Histone covalent modification. HAT's and HDAC's are able to change the association of DNA with histones, allowing for genes to be active or repressed (adapted from (Weaver 2002)

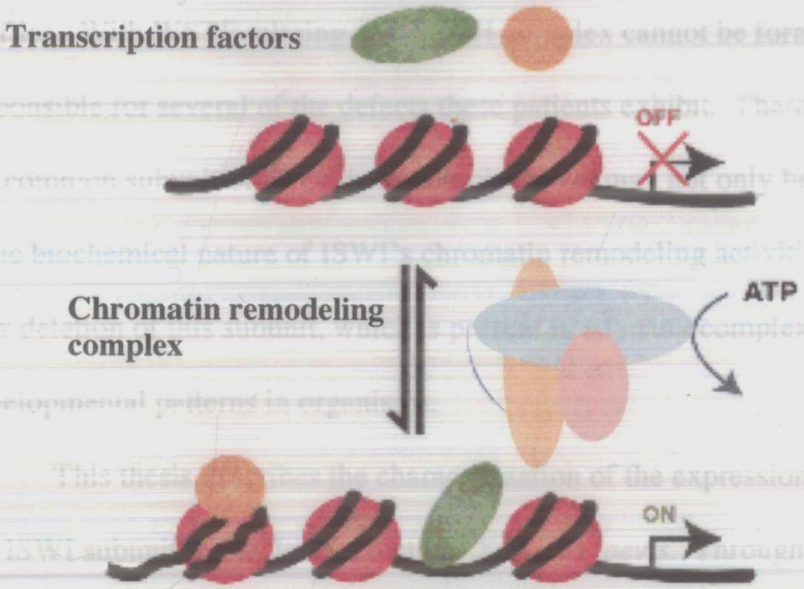

Figure 1.5 ATP-dependent chromatin remodeling. The multisubunit complexes of ATP-dependent chromatin remodelers use the power of ATP hydrolysis to move histones to repress or activate gene transcription. This came from a recent review on remodelers, have to find out which one (adapted from http://faculty.jsd.claremont.edu/jarmstrong/researchint.htm).

profiles. With WSTF missing, the WICH complex cannot be formed; this loss may be responsible for several of the defects these patients exhibit. Therefore, by investigating the common subunit of all of these complexes we may not only be able to understand the basic biochemical nature of ISWI's chromatin remodeling activities but also discover how deletion of this subunit, which is present in so many complexes, may alter developmental patterns in organisms.

This thesis describes the characterization of the expression pattern and function of the ISWI subunit in the development of *Xenopus laevis*. Through *in situ* hybridization I have shown which tissues express *ISWI* mRNA throughout different developmental stages. I have used microinjection of antisense *ISWI* RNA, anti-ISWI morpholino or dominant-negative ISWI mutants to explore the effects of a nonfunctional or absent ISWI protein. Because ISWI is a subunit in at least four different *Xenopus* ATP-dependent chromatin remodeling complexes, I also examined the expression pattern of specific mRNAs that are potential targets for ISWI-dependent remodeling. I also tested direct DNA binding by ISWI protein using chromatin immunoprecipitation (ChIP). With these experiments I have begun to establish the critical role of ISWI in the developing *Xenopus laevis* embryo.

Chapter 2

The SWI2/SNF2 subfamily

At least 7 distinct subfamilies of the SWI2/SNF2 superfamily have been found to date. These families include SNF2, CHD1, INO80, CSB, RAD54, DDM1 and Imitation Switch (ISWI) (Lusser and Kadonaga 2003). Proteins from each of these subfamilies have been found to participate a wide array of ATP-dependent chromatin remodeling processes such as transcriptional activation and repression, nucleosome spacing or sliding, replication, repair, recombination and nucleosomal assembly. Next we will discuss the unique properties to all of these subfamilies.

The CHD family

The CHD subfamily, like all members of the SWI2/SNF2 superfamily, contains a conserved ATPase domain. However, these family members also contain a chromo (chromatin organization modifier) domain and a DNA binding domain. Chromo domains are found in a number of proteins, including many that have the ability to interact with heterochromatin. Chromo domains have been found to act as recognition motifs for methylated lysine 9 of H3 (Lachner et al. 2001; Nakayama et al. 2001). Chromo domains have also been shown to interact with RNA as well as to self associate with one another. CHD complexes have been found in many organisms including yeast, human, *Drosophila* and *Xenopus*.

In the budding yeast *S. cerevisiae* there is only one CHD protein, Chd1. Chd1 purification shows that it does not associate with other proteins in a complex, but elutes

from sizing columns at double its molecular weight, indicating that it may exist as a dimer (Neely and Workman 2002). *chd1* null mutants are viable; however, a *chd1/swi2* double mutant is synthetically lethal suggesting that the Chd1 and SWI/SNF complexes may have overlapping functions (Tran et al. 2000). Likewise, fission yeast (*Schizosaccharomyces pombe*) contain only one CHD family member, Hrp1 , characterized by a chromodomain, a Myb-like telobox-related DNA-binding domain and a SWI2/SNF2 ATPase domain (Jin et al. 1998).

In humans many complexes contain a CHD family member and contain ATP-dependent nucleosome remodeling activities. NURD (aka NuRD and NRD) was discovered and named in three separate laboratories and contains both ATP-dependent remodeling and histone deacetylase activity (Tong et al. 1998; Xue et al. 1998; Zhang et al. 1998). CHD4/Mi-2β and CHD3/Mi-2α are highly related proteins that were identified as autoantigens in the human disease dermatomyositis, and provide the ATP-dependent chromatin remodeling activity of NURD. There has been no recent data indicating whether Mi-2α and Mi-2β are in the same complex or exist in separate but related complexes (Neely et al. 2002; Bowen et al. 2004). Although both appear to present in the same tissues, Mi-2β seems to be the more abundant isoform to elute with the other subunits of NURD (Tong et al. 1998). Mi-2α elutes from phosphocellulose fractionation at a separate salt fraction as well as the same salt fraction as Mi-2β. The Mi-2α fraction that does not elute with NURD does retain its HDAC function suggesting Mi-2α may have a unique function in an alternative complex or alone yet to be discovered (Schultz et al. 2001).

Shultz et al. (2001) also identified a domain unique to the Mi-2α isoform known as the KRAB domain. The KRAB domain is a highly conserved domain, which displays potent, DNA-binding-dependent repression of transcription that requires the KAP-1 co-repressor. This finding agrees with the current models, which predict the NURD complex functions primarily in transcriptional repression. Both Mi-2α and Mi-2β associate with many other proteins within the NURD complex including: histone deacetylases (HDAC1 & 2), Rb-associated proteins (RbAp48 & 46), metastasis associated proteins (MTA1 & 2) and the MBD protein family, which contain the methyl-CpG binding domain (Tong et al. 1998; Xue et al. 1998; Zhang et al. 1998; Wang and Zhang 2001). Uniquely, all of the subunits of the NURD complex present heterogeneity at the protein and gene level, raising the possibility for specialization as different isoforms of each subunit are recruited.

In *Xenopus* a complex highly homologous to the NURD complex was isolated from egg extracts (Wade et al. 1998; Wade et al. 1999). This complex contains Rpd3, a histone deacelylase, and the Mi-2 ATPase. Other components include RbAp48/p46, MTA1-like, MBD3 and MBD3 LF (long form). One contrast to human NURD is that MBD3 in *Xenopus* NURD is able to target and bind methylated DNA, whereas human NURD cannot (Bowen et al. 2004).

Drosophila also contains a Mi-2 homolog named dMi-2 (Brehm et al. 2000). dMi-2 exists in a large complex, similar to its human and *Xenopus* counterparts, but this complex is not well characterized. It does contain dRpd3 and histone deacetylase activity; however, dMi-2's ATPase activity is only stimulated by nucleosomes (Brehm et al. 2000)

dMi-2 was shown to have the ability to bind nucleosome cores (which presumably display no free DNA) and dMi-2 moves histone octamers in one direction in sliding assays suggesting it uses a unique mechanism of nucleosome mobilization (Brehm et al. 2000).

The INO80 family

The Ino80 complex in yeast is the most recently described chromatin remodeling complex and is referred to as Ino80.com (Shen et al. 2000). The Ino80 complex remodels chromatin, facilitates transcription in vitro, and displays ATP-dependent chromatin remodeling activity and in yeast it is known to be associated with chromosomes of dividing cells (Shen et al. 2000). The *INO80* gene from yeast forms a distinct subfamily, which has not yet been well characterized. Null mutants show not only hypersensitivity to alkylating agents and ultraviolet as well as ionizing radiations, but they also have defects in transcription (Ebbert 1999; Shen et al. 2000). Ino80 mutants in yeast have been shown to be hypersensitive to DNA damaging agents and to double strand breaks induced by the HO endonuclease through a specific interaction with the DNA-damage induced phosphorylated histone H2A (Morrison et al. 2004; van Attikum et al. 2004).

Ino80 has both *Drosophila* and human homologs. The INO80 family was recently characterized in silico and was found to lack a SANT domain and a bromodomain; however, sequence alignments revealed that these proteins contain a conserved region beyond the seven motif ATPase domain (Bakshi et al. 2004). This domain was found in all homologs of INO80, including mouse, human, *Drosophila*, yeast and plants (Bakshi et al. 2004). All family members analyzed contained what is now referred to as a DBINO

(DNA Binding INO80) domain. This domain is located in the N-terminus upstream of the ATPase domain and consists of 126 amino acids. Due to the high incidence of arginine and lysine, which are two positively charged amino acids, this site was deemed a putative DNA binding domain. In support of this, Shen et al. reported a detailed study on the role of actin-related protein (ARP) complex in yeast. They found that when a 326 amino acid region in the N-terminus was deleted there was a loss of DNA binding activity. This proved interesting because the DBINO domain maps to this region (Bakshi et al. 2004). Ino80 was purified as a complex with twelve associated proteins. Actin (Act1) and three other actin-related proteins (Arp4, Arp5, Arp8) were found to co-purify with INO80. Additionally, two helicase proteins, Rvb1 and Rvb2, were identified. The other 5 proteins have not been identified to date, but are present in equal amounts.

The SNF2 family

The SNF2 subfamily includes the yeast Swi2/Snf2 subunit of the SWI/SNF complex, the yeast Sth1 subunit of the RSC complex, and the *Drosophila* Brahma, human BRG1 and hBRM subunits of the SWI/SNF-related complexes in *Drosophila* and humans. SNF2 subfamily members contain a bromodomain in addition to the ATPase region.

Mammalian SWI/SNF contains one of two ATPases, BRM and BRG1 along with variable subunit composition of BRG1-associated factors (BAFs). BRG1 and BRM are highly homologous ATPases, yet they appear to direct very different cellular pathways. Gene knockout studies in mice have demonstrated that homozygous inactivating mutations in BRG1 are embryonic lethal whereas BRM-inactivated mice are mainly

characterized as having excess body weight (Reyes et al. 1998; Bultman et al. 2000). BRG1 and BRM activities are partially redundant but they have different expression profiles. BRG1 protein levels are relatively consistent in all cells whereas BRM protein concentrations increase during cellular differentiation (Wang et al. 1996). A variety of human malignancies are associated with mutations in BRG1, but not BRM, suggesting that a loss of function of certain SWI/SNF complexes may contribute to tumorigenesis (Klochendler-Yeivin et al. 2002). BRG1 and BRM interact specifically with different classes of regulatory proteins. BRG1 but not BRM functions with zinc finger proteins (ZFP) and recruits them to specific chromatin sites (Kadam and Emerson 2003). Either BRG1 or BRM, but not both, interact with different SWI/SNF-responsive promoters, supporting the divergence of function for these two homologs. The BRM ATPase is expressed at high levels in differentiating cells, but the functional role is not understood. Kadem et al. (2003) found that two components of the Notch pathway associate with BRM not BRG1. The Notch pathway controls cell fate commitment in a broad range of developmental pathways. BRG1-containing SWI/SNF is involved in regulating genes induced by cytokine pathways through interferon α or γ (Liu et al. 2002; Pattenden et al. 2002). A common feature of the BRG1/BRM target genes is that they promote cellular differentiation and may have evolved to counter the cellular demands of higher eukaryotes. BRG1 and BRM have been recently shown in cell culture to be required for estrogen antagonist-mediated growth suppression through the estrogen receptor, which suggest that BRG1 and BRM may be major cellular targets for estrogen antagonists, which are potentially important targets for breast cancer therapy (Wang et al. 2004).

BRM and BRG1 have been implicated as possible prognostic indicators for non-small cell lung cancer (Fukuoka et al. 2004).

The essential Sth1p is the protein most closely related to Snf2p/Swi2p in *Saccharomyces cerevisiae*. Sth1p purified from yeast has a DNA-stimulated ATPase activity required for its function in vivo (Haynes et al. 1992; Laurent and Carlson 1992; Tamkun et al. 1992; Jeanmougin et al. 1997). Sth1p is the catalytic component of a multiprotein complex capable of ATP-dependent remodeling of the structure of chromatin (RSC). Three *sth1* temperature-sensitive mutations map to the highly conserved ATPase domain and have cell cycle and non-cell cycle phenotypes, suggesting multiple essential roles for Sth1p (Du et al. 1998). The Sth1p bromodomain is required for wild-type function; deletion mutants lacking portions of this region are thermo-sensitive and arrest with highly elongated buds and 2C DNA content, indicating perturbation of a unique function (Du et al. 1998). The pleiotropic growth defects of *sth1*-ts mutants imply a requirement for Sth1p in a general cellular process that affects several metabolic pathways. Significantly, an *sth1*-ts allele is synthetically sick or lethal with previously identified mutations in histones and chromatin assembly genes that suppress *swi/snf* mutations, suggesting that RSC interacts differently with chromatin than SWI/SNF (Du et al. 1998). These results provide a framework for understanding the ATP-dependent RSC function in modeling chromatin and its connection to the cell cycle.

Drosophila has only one Swi2/Snf2 family member called Brahma (BRM) (Dingwall et al. 1995; Papoulas et al. 1998). The BRM complex is composed of approximately eight proteins, which are referred to as BAPs (BRM-associated proteins).

These proteins include the trithorax group (trxG), Moria/Bap155 (MOR), OSA/Eyelid and Snr1, Bap60, Bap55, Bap111, β-actin/Bap47 and BAP74 (Dingwall et al. 1995; Crosby et al. 1999; Collins and Treisman 2000; Kal et al. 2000). The *Drosophila* BRM complex was identified on the basis of its requirement for the maintenance of homeotic (HOM) gene expression (Tamkun 1995; Elfring et al. 1998), and is essential for proper development. Mutations in several BRM complex genes give rise to a broad range of developmental defects (Tamkun 1995).

The RAD54 family

Rad54 and its relation to the SWI2/SNF2 superfamily was first analyzed in yeast. Since then other homologs in yeast and human have been identified. The family members of the RAD54 subfamily include RAD54, ATRX and ARIP4 (Lusser and Kadonaga 2003). Each homolog has been implicated in unique cellular activities ranging from homologous recombination of double strand breaks to transcription.

The *RAD54* gene plays an important role in recombination and DNA double strand break repair in *Saccharomyces cerevisiae* and in humans. In vitro, Rad54 interacts with Rad51 and stimulates DNA strand exchange promoted by Rad51 protein. Rad54 is a SWI2/SNF2-related protein that possesses double-stranded DNA-dependent ATPase activity and changes DNA topology in an ATP hydrolysis-dependent manner (Alexeev et al. 2003). Rad54 catalyzes bidirectional nucleosome redistribution by sliding nucleosomes along DNA (Alexeev et al. 2003). There are two RAD54-homologous genes in human cells, hRAD54 and RAD54B. Point mutations in these human genes have been found in tumors. These tumor-associated mutations map to conserved regions of the

hRad54 and hRad54B proteins. Equivalent mutations were introduced with site directed mutagenesis into the *Saccharomyces cerevisiae RAD54* gene to explore the effects of these mutations (Smirnova et al. 2004). One mutant, rad54-G484R, showed sensitivity to DNA-damaging agents and reduced homologous recombination rates, indicating a loss of function. Purified rad54-G484R mutant protein retained the ability to bind DNA and interact with Rad51, but it was nearly devoid of ATPase activity. Two other mutants, rad54-N616S and rad54-D442Y, were not sensitive to genotoxic agents and behaved like the wild type allele in homologous recombination assays (Smirnova et al. 2004). This suggests that the tumor phenotype may be enhanced by the enhanced genomic instability of tumor cells lacking RAD54 function.

The ATRX protein was first identified in patients with ATRX syndrome. Mutations in the ATRX gene cause several X-linked mental retardation syndromes, which consist of the following phenotypes: facial dysmorphism, urogenital defects, and α-thalassaemia (Gibbons et al. 2000). However, until recently, the ATRX protein had not been biochemically characterized. ATRX was first thought to have ATP-dependent activity due to the sequence homology to SWI/SNF2. ATRX contains an ATPase/helicase motif as well as a plant homeodomain-like zinc finger (Villard et al. 1997), both of which have been found in molecules that modify chromatin structure. ATRX has also been found in nuclear extracts and is a protein of about 280 kDa (Berube et al. 2002). ATRX localizes at pericentromeric heterochromatin (McDowell et al. 1999) and has been identified in yeast two-hybrid screens to interact with the heterochromatin protein HP1 as well as a polycomb group protein EZH2 (Cardoso et al. 1998; McDowell

et al. 1999). Mutations in ATRX have been correlated with changes in DNA methylation patterns at several genomic loci (Gibbons et al. 2000). ATRX is now known to form a complex with a transcription cofactor, Daxx, and this complex displays chromatin-remodeling activities (Xue et al. 2003).

Recently a novel ATP-dependent chromatin remodeler has been characterized by Rouleau et al (2002) that belongs to the SNF2 family and RAD54 subfamily of proteins. The protein was termed ARIP4 (androgen receptor interacting protein 4). ARIP4 interacts with androgen receptors (AR) in vivo and in vitro. It generates super-helical torsion within linear DNA fragments in an ATP-dependent manner and it modulates AR-mediated transcription (Rouleau et al. 2002). ARIP4 mutants incapable of hydrolysis fail to alter DNA topology and lose the ability to activate AR-dependent transcription (Rouleau et al. 2002). These mutants also behave as trans-dominant negative regulators of AR function when expressed ectopically in transient transfection assays (Rouleau et al. 2002).

The CSB family

The Cockayne Syndrome B (CSB) subfamily belongs to the Swi2/Snf2 family of DNA-dependent ATPases (CSB, Rad26). These proteins enable transcription-coupled repair (TCR). This form of DNA repair occurs on the transcribed strand so that transcription is not stalled at an error. The human disease Cockayne Syndrome (CS) is associated with a defect in TCR. The CSA and CSB (aka ERCC6) genes have been identified as uniquely affecting TCR in mammalian cells. A yeast gene with homology to the human CSB gene (*RAD26*) was isolated and sequenced (Huang et al. 1994; van

Gool et al. 1994). Next a *rad26* deletion mutant was created(van Gool et al. 1994). Consistent with the DNA repair deficiency observed in cultured cells from CS patients, the yeast *rad26Δ* mutant lacks TCR activity (van Gool et al. 1994). Previous repair rates for the *rad26Δ* mutant have all been determined in one genetic background, W303-1B. Recently a deletion of *RAD26* in three other strains of different genetic backgrounds was carried out and their ability to carry out TCR was examined by Gregory et al. (2001). Surprisingly, no defect was observed in TCR of an expressed gene in these new *rad26Δ* mutants. Notably, deletion of *RAD26* enabled expression from genes flanked by δ elements, δ elements alter local chromatin structure and gene expression (Gregory and Sweder 2001). Suppression of δ element phenotypes suggests a role for Rad26 in chromatin remodeling or transcription elongation. Gregory et al. suggested that these results implicate Rad26 (or CSB) to be necessary but not sufficient to enable TCR.

The DDM1 family

Deficient in DNA Methylation 1 (DDM1) protein is required to maintain the DNA methylation status of *Arabidopsis thaliana.* Methylation of cytosine residues in the genome is thought to be crucial for normal development in mammals as well as plants, frogs, and fish. DNA methylation is involved in the regulation of a diverse range of biological processes such as genomic imprinting and X-chromosome inactivation (Yeivin and Razin 1993; Razin and Shemer 1995). DDM1 is a member of the broad SWI2/SNF2 protein family and is conserved in plants, yeast and mammals. No genes encoding DDM1-like proteins have been found in the two fully sequenced invertebrate genomes. Evolutionary analysis places the DDM1 subfamily close to ISWI and SNF2/SWI2 groups

(Verbsky and Richards 2001). Previously it was unclear how DDM1 acts to maintain DNA methylation status, although it had been speculated that it could remodel chromatin. Brzeski et al. (2003) found that DDM1 is an ATPase stimulated by both naked and nucleosomal DNA. It binds to nucleosomes and promotes nucleosome repositioning in an ATP-dependent manner (Brzeski and Jerzmanowski 2003). This data indicated that DDM1 defines a novel class of chromatin-remodeling factors.

LSH (lymphoid specific helicase) is another member of the DDM1 subfamily. LSH shows a preferential lymphoid expression pattern in adult mice and has been shown to be important for normal lymphoid development (Geiman et al. 1998; Geiman and Muegge 2000). A low expression of LSH has been found in multiple embryonic tissues suggesting a broader role for LSH in development (Geiman et al. 2001). LSH shares about 50% identity with DDM1 over the region containing the helicase domains (Jeddeloh et al. 1999). A recent study investigated the effect of LSH on genomic methylation patterns in mice. Based on LSH's presumed chromatin remodeling activity it was speculated that LSH may regulate chromatin accessibility for DNA methyltransferases. Because LSH protein expression correlates with S-phase of the cell cycle (Geiman and Muegge 2000), LSH may facilitate access of DNA methyltransferases to hemimethylated sites after replication occurs and thus co-operate to maintain methylation patterns. Alternatively, the presence of LSH may protect against demethylase activities (Jarvis et al. 1996; Ramchandani et al. 1999). Methylation of histone tails and CpG methylation are involved in determining heterochromatin structure, LSH has recently been reported to control both types of epigenetic modifications (Yan et al. 2003).

Loss of LSH in mice results in accumulation of di- and tri-methylated histone 3 at lysine 4 (H3-K4me) at pericentromeric DNA and other repetitive sequences (Yan et al. 2003). LSH is associated with pericentromeric heterochromatin and is be required for normal CpG methylation at pericentromeric sequences (Yan et al. 2003). LSH seems to be crucial for the formation of normal heterochromatin, which implies a role for LSH in the regulation of transcription and mitosis.

The ISWI Family[1]

The ISWI family of ATPases is distinguished from the other SNF2/SWI2-related subfamilies by the presence of SANT (SWI3, ADA2, N-CoR, TFIIIB) domains in the C-terminal half of the protein. The ISWI group was originally identified in *Drosophila*, from which three different ISWI-based remodeling complexes were purified: NURF (nucleosome remodeling factor), ACF (ATP-depdendent chromatin-assembly and -remodeling factor) and CHRAC (chromatin accessibility complex) (Becker et al. 1994; Tsukiyama et al. 1994; Tsukiyama et al. 1995; Tsukiyama and Wu 1995).

ISWI-based nucleosome-remodeling complexes have since been identified in yeast, *Xenopus*, *Arabidopsis*, and mammals. In yeast, two closely related ISWI proteins, Isw1p and Isw2p, are present in four different complexes: Isw1a, Isw1b, Isw2, and Isw2/yCHRAC (Tsukiyama et al. 1999; Vary et al. 2003; Iida and Araki 2004). ISWI protein is present in four distinct biochemical fractions in *Xenopus* oocytes (Guschin et al. 2000). Two of these ISWI-containing complexes are homologs of the ACF and

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[1] This section is a modified version of a published review: Dirscherl, S. and Krebs, JE. (2004). "Functional diversity of ISWI complexes." Biochem Cell Biol. **82**(4): 482-9. The complete publication is found in Appendix A.

CHRAC complexes. A third complex is the WICH complex, a complex of ISWI and the Williams Syndrome Transcription Factor (WSTF) (Bozhenok et al. 2002). The fourth ISWI complex has not been characterized, and a *Xenopus* homolog of NURF has not been identified (Guschin et al. 2000). In mammals, two related ISWI homologs are encoded by the *SNF2L* and *SNF2H* genes. The SNF2H subunit has been found in a remarkable six different complexes. These include RSF (remodeling and spacing factor) (LeRoy et al. 1998; Loyola et al. 2003), hACF/WCRF (WSTF-related chromatin remodeling factor) (Bochar et al. 2000; LeRoy et al. 2000), hWICH (Bozhenok et al. 2002), hCHRAC (Poot et al. 2000), and NoRC (nucleolar remodeling complex) (Strohner et al. 2001). Finally, SNF2H has also been identified in a complex that also contains cohesin and subunits of the NuRD complex, a nucleosome remodeling and histone deacetylase complex containing the Mi-2 ATPase (a member of the CHD1 subfamily) (Hakimi et al. 2002). SNF2L has recently been identified as the catalytic subunit of human NURF (Barak et al. 2003). The first ISWI homolog in *Arabidopsis*, PIE1, has recently been identified (Noh and Amasino 2003), but has not yet been characterized biochemically.

The functional diversity of ISWI complexes is nearly as great as the sheer number of complexes performing these functions. *In vivo* functions attributed to ISWI complexes include transcriptional activation and repression, chromatin assembly, nucleosome spacing or sliding, replication through heterochromatin, maintenance of higher order chromatin structure, nuclear remodeling of somatic cells, and loading of cohesin complex. These different *in vivo* functions are summarized in table I, and are detailed

below in the descriptions of specific complexes (Tsukiyama 2002). The mechanism of ISWI-dependent remodeling has also been extensively studied in vitro. These studies have been well-reviewed elsewhere (Langst and Becker 2001b; Becker and Horz 2002; Peterson 2002; Flaus and Owen-Hughes 2003; Lusser and Kadonaga 2003; van Holde and Yager 2003), and will only be briefly addressed here. The known subunit compositions of ISWI complexes are depicted schematically in figure 1. In the following sections, I have grouped the various ISWI complexes by homology of species and related *in vivo* functions.

Chromatin assembly and replication: ACF, CHRAC, RSF, WICH

Among the first ISWI complexes purified were the ACF and CHRAC complexes from *Drosophila*, which exhibit the ability to assemble nucleosomes into regularly spaced nucleosomal arrays (Ito 1997; Ito et al. 1997; Varga-Weisz et al. 1997). These complexes are closely related; ACF contains only ISWI and ACF1 (Ito et al. 1999), and CHRAC is essentially ACF plus two other subunits, CHRAC-14 and CHRAC-16 (Corona et al. 2000). CHRAC-14 and CHRAC-16 contain histone fold domains and are conserved in mammalian (CHRAC-15 and CHRAC-17) and *Xenopus* (CHRAC-17) CHRAC (Guschin et al. 2000; Poot et al. 2000). Human CHRAC-15/17 has been shown to facilitate the in vitro nucleosome sliding activity of ACF-ISWI (Kukimoto et al. 2004). The chromatin assembly factor RSF, like ACF, is a small complex consisting of only two

Table 2.1 *In vivo* functions of ISWI-containing complexes. (ACF/CHRAC complexes have *in vitro* assembly/spacing functions, not all have been tested). Details and references are contained in the text.

Complex	Organism	# sub-units	Subunits	In vivo function(s)
dACF	*Drosophila*	2	ISWI and ACF1	Assembly/spacing
xACF	*Xenopus*	3	ISWI, xACF1, p175	Assembly/spacing
hACF	Human	2	SNF2h and ACF1/WCRF180	Assembly/spacing
yCHRAC	Yeast	4	Isw2p, Itc1p, Dpb4p, Dls1p	Telomere position effect/heterochromatin structure
dCHRAC	*Drosophila*	4	ISWI, ACF1, CHRAC14, CHRAC16	Assembly/spacing
xCHRAC	*Xenopus*	5	ISWI, p200, p70, p55 and CHRAC17	Assembly/spacing
hCHRAC	*Human*	4	hSNF2H, hACF1, p15 and p17	Assembly/spacing
RSF	*Human*	2	hSNF2H and Rsf-1/p325	Assembly/spacing
xWICH	Xenopus	2	ISWI and WSTF	Heterochromatin replication, transcription
hWICH	*Human*	2	SNF2H and WSTF	Heterochromatin replication, transcription
yISW1a	Yeast	2	Isw1p and Ioc3p	Transcription repression (many genes)
ISW1b	Yeast	3	Isw1p, Ioc2p, Ioc4p	Transcription elongation and termination
ISW2	Yeast	2	Isw2p and Itc1p	Transcription repression (many genes), nucleosome sliding
dNURF	*Drosophila*	4	ISWI, p301, NURF-55, NURF-38	Transcription activation (heat shock and homeotic genes)
hNURF	Human	4	SNF2L, BPTF and RbAP46/48	Transcription activation (*engrailed*)
NoRC	Human	2	SNF2h and Tip5	rDNA repression
PIE1	*Arabidopsis*	?	PIE1 and ?	Transcription activation (FLOWERING LOCUS C)
SNF2h/ NuRD/ cohesin	Human		SNF2h, NuRD (Mi-2, HDAC1/2, MBD2/3, MTA1/2, RbAp46/48), cohesin (hRAD21, hSMC1/2, SA1/SA2)	sister chromatid cohesion

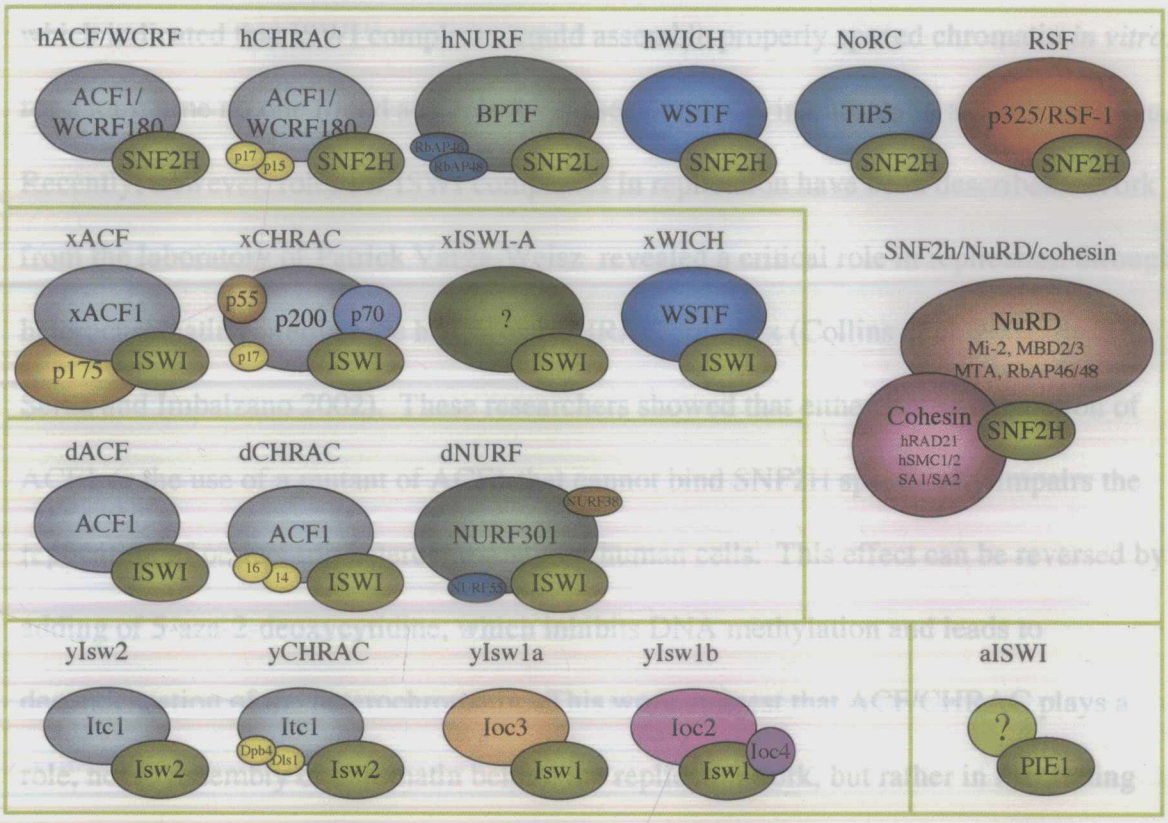

Figure 2.1 Subunit compositions of ISWI complexes. Colors indicate homologous proteins. Drawings are not to scale and only reflect relative sizes of subunits. No specific subunit interactions or complex substructures are implied. Details are found in the text.

subunits, SNF2H and Rsf-1/p325 (LeRoy et al. 1998; Loyola et al. 2003). Thus far, RSF has only been identified in human cells. RSF can assemble and space chromatin *in vitro*, and, unlike ACF and CHRAC, does not require histone chaperones, because RSF performs histone-chaperone activity itself (Loyola et al. 2001). Despite these studies, which indicated that ISWI complexes could assemble properly spaced chromatin *in vitro*, for a long time no one found any role for these complexes in chromatin assembly *in vivo*. Recently, however, roles for ISWI complexes in replication have been described. Work from the laboratory of Patrick Varga-Weisz revealed a critical role in replication through heterochromatin for either the hACF or hCHRAC complex (Collins et al. 2002; de la Serna and Imbalzano 2002). These researchers showed that either RNAi-depeletion of ACF1 or the use of a mutant of ACF1 that cannot bind SNF2H specifically impairs the replication of pericentric heterochromatin in human cells. This effect can be reversed by adding of 5-aza-2-deoxycytidine, which inhibits DNA methylation and leads to decondensation of the heterochromatin. This work suggest that ACF/CHRAC plays a role, not in assembly of chromatin behind the replication fork, but rather in facilitating movement of the fork through dense heterochromatin.

Recent work in the Kadonaga laboratory indicates that Drosophila ACF/CHRAC complexes are required for proper chromatin assembly, particularly repressive chromatin structures (Fyodorov et al. 2004). Only 25% of Acf-null flies survived past the pupal stage; these survivors exhibited reduced nucleosome periodicity and reduced average nucleosomal spacing in bulk chromatin in vivo. Further, loss of Acf1 resulted the loss of transcriptional silencing in pericentric heterochromatin and *Polycomb*-repressed

chromatin, suggesting that ACF/CHRAC plays a key role in establishing or maintaining repressive chromatin. Acf1-deficient embryos also exhibited a shortened S phase, possibly resulting from the lack of chromatin repression of DNA replication.

In contrast, depletion of ISWI from *Xenopus* egg extracts does not appear to affect the efficiency of either replication or histone deposition, although the nucleosome spacing of the resulting chromatin is disturbed (Demeret et al. 2002; MacCallum et al. 2002). However, protein-free DNA and sperm chromatin, two common templates for remodeling and replication in egg extracts, are very different substrates from the native chromatin described in the previous experiments (Collins et al. 2002), so it is difficult to compare the results directly.

The WICH complex, found in both *Xenopus* and mammals, consists of ISWI (SNF2H) and WSTF, and has been linked both to replication and transcription. In mouse cells, WICH is localized to pericentric heterochromatin during replication (Bozhenok et al. 2002), in the same way that ACF1 and SNF2H are localized in human cells (Collins et al. 2002). WSTF is related to ACF1 (also known as WCRF180, WSTF-related chromatin remodeling factor) (Bochar et al. 2000), but the functional differences between ACF and WICH complexes are not clear. In *Xenopus*, WICH also binds stably to mitotic chromosomes, clearly distinguishing it from the S-phase chromatin binding of ACF (Bozhenok et al. 2002). In addition, WSTF itself may also occur in a complex with SWI/SNF in human cells (Kitagawa et al. 2003).

A potential yeast homolog of CHRAC has recently been described (Iida and Araki 2004). This complex is related to the previously characterized Isw2 complex, which

consists of Isw2p and Itc1p (Tsukiyama et al. 1999), but contains two additional small subunits: Dpb4p, a histone-fold motif-containing subunit of the replicative DNA polymerase ε; and Dls1p, which is related to the histone-fold partner of Dpb4p, Dpb3p. Dpb4p is the yeast homolog of the CHRAC-17 subunit (which is also a component of human Pol ε). yCHRAC has also been shown to have functional links to replication. Pol ε and yCHRAC have opposing effects on epigenetic silencing at the telomere, with Pol ε promoting silencing and yCHRAC promoting expression of subtelomeric genes. This suggests that the interplay between Pol ε and yCHRAC (perhaps mediated by their common subunit, Dpb4p) controls the maintenance or resetting of the epigenetic state of the telomeres during replication.

Transcriptional regulation: NURF, NoRC, Isw1, Isw2

The first ATP-dependent remodeler to be characterized, yeast SWI/SNF, was identified genetically as a positive regulator of transcription (Hirschhorn et al. 1992; Peterson and Herskowitz 1992; Laurent et al. 1993; Peterson et al. 1994). *In vitro*, all of the SWI2/SNF2 superfamily-containing complexes are able to make DNA in chromatin accessible to site specific binding factors, such as transcription factors or restriction enzymes (Imbalzano et al. 1994; Kwon et al. 1994; Logie and Peterson 1997; Boyer et al. 2000; Fan et al. 2003). For years, this work seemed to foster the belief that all chromatin remodelers would turn out to be transcriptional activators. However, some early work clearly indicated that chromatin remodelers could also be involved in repression. The human SWI2/SNF2 family members hbrm and Brg1 have been shown to work as corepressors with RB (retinablasoma) (Trouche et al. 1997; Murphy et al. 1999), and the

appearance of the SWI2/SNF2 homolog Mi-2 in complexes with histone deacetylases raised the idea that remodeling might also be involved in transcriptional repression (Zhang et al. 1998). Since then it has become clear that chromatin remodeling can result in activation or repression of transcription in vivo. This is discussed below in detail for ISWI.

ISWI family complexes appear to both activate and repress transcription. The first role for an ISWI complex in transcription came from work in the Wu laboratory on the NURF complex (Tsukiyama et al. 1994; Tsukiyama et al. 1995; Tsukiyama and Wu 1995; Mizuguchi et al. 1997). In addition to ISWI, NURF contains three other protein subunits: NURF55, NURF38 (an inorganic pyrophosphatase) and a large NURF301 subunit (Gdula et al. 1998; Martinez-Balbas et al. 1998). Work *in vivo* has shown that *nurf301* and *iswi* mutations result in impaired transcription of heat shock genes (*hsp70* and *hsp26*) and homeotic genes (*ubx* and *en*) (Deuring et al. 2000; Badenhorst et al. 2002).

The recently isolated human NURF complex, the only known SNF2L-based complex, is also involved in transcriptional activation. In fact, depletion of SNF2L using RNAi results in the reduced expression of the human *engrailed* genes *en-1* and *en-2*, homologs of the *Drosophila en* gene that requires NURF for its expression (Barak et al. 2003).

In contrast, other ISWI complexes are primarily involved in transcriptional repression. The NoRC complex, the mammalian nucleolar remodeler, is composed of SNF2H and TIP5, a protein originally identified as a partner of the RNA Pol I

termination factor TTF-1 (Strohner et al. 2001). TTF-1 is also somewhat related to WSTF and Acf1/WCRF180, providing a link between multiple ISWI complexes. NoRC has since been shown to be involved in repression of Pol I transcription, through the recruitment of the SIN3/HDAC1 corepressor complex to the rDNA promoter (Zhou et al. 2002).

In yeast, Isw1a, Isw1b, Isw2 have all been implicated in transcriptional repression. The Isw2 complex is recruited by the general repressor Ume6p, and represses early meiotic genes (Goldmark et al. 2000), *INO1*(Kent et al. 2001), a-cell specific genes in α-cells (Ruiz et al. 2003), and a variety of metabolic, stress-responsive, and other genes (Fazzio et al. 2001). Likewise, deletion of components of the Isw1 complexes suggests that Isw1 plays a primary role in transcriptional repression, measured by whole genome expression analysis (Vary et al. 2003).

Other studies of the Isw1 complexes have revealed that they play a role in a different aspect of transcriptional regulation: elongation and termination (Alen et al. 2002; Morillon et al. 2003). Morillon et al. (2003) proposed that Isw1p sequentially regulates each stage of the transcription cycle, linking events at the 5' and 3' end of the transcription unit and controlling the amount of RNAPII entering productive elongation. In fact, these studies appear to have separated the roles of Isw1a and Isw1b: Isw1a (Isw1p + Ioc3p) acts as a repressor and prevents transcription initiation, while Isw1b (Isw1p, Ioc2p, Ioc4p) appears to control elongation, coordinating Pol II CTD phosphorylation with events involved in RNA 3' end formation, and may also promote release of Pol II during terminantion (Morillon et al. 2003). The ability of Isw1to regulate

transcription of some genes is linked to Set1p-dependent methylation of histone H3 *in vivo* (Santos-Rosa et al. 2003).

Higher-order chromosome structure: ISWI/cohesin, ISWI-?

Mitotic (or meiotic) chromatin, the most highly condensed state of eukaryotic DNA, represents an extra challenge for any factors that require access to the DNA. Some genes in yeast appear to require more remodeling to be expressed during mitosis than during other stages of the cell cycle (Krebs et al. 2000). Chromosomes also exhibit unique behaviors during mitosis and meiosis; one of the most striking being the cohesion of sister chromatids before anaphase, and their abrupt release at the metaphase-to-anaphase transition (Haering and Nasmyth 2003; Hagstrom and Meyer 2003; Morrison et al. 2003). It should come as no surprise that unique structures of mitotic and meiotic chromosomes also depend on chromatin remodelers.

In the case of sister chromatid cohesion, cohesin loading appears to require a complex of complexes (Hakimi et al. 2002). Human SNF2H is associated not only with the core cohesin complex (hRAD21, SMC1, SMC3 and SA1/SA2), but also with the NuRD complex, which contains Mi-2 (another SWI2/SNF2 family ATPase), the methyl-DNA-binding proteins MBD2 and -3, HDAC1 and -2, the metastasis-associated proteins MTA1 and -2, and the Rb-associated proteins RbAp46 and -48 (Feng and Zhang 2003). These associations, identified using extensive biochemical fraction and affinity chromatography, are also reflected *in vivo* in chromatin immunoprecipitation (ChIP) experiments that show colocalization of SNF2H, NuRD and cohesion at multiple Alu sequences. This colocalization also correlates with histone H3K4 methylation and H3/H4

acetylation (which implies that there is little active deacetylation occurring from the NuRD complex in this context). Importantly, the association between cohesin and chromatin requires a catalytically active SNF2H, suggesting that the remodeling function of SNF2H is critical for cohesin loading.

ISWI complexes in other species have not yet been associated with sister chromatid cohesion, but they have been implicated in similarly large-scale chromatin events. *ISWI* and *NURF301* mutations in *Drosophila* have dramatic and global effects on the structure of the male X chromosome in polytene chromosomes (Deuring et al. 2000; Badenhorst et al. 2002). The X chromosomes are significantly shorter and broader in males, whereas the autosomes in both males and females tend to be thinner than normal in the *ISWI* mutants. Polytene chromosomes are generated by multiple cycles of endoreplication (replication without division). The observed effects could be due to a replication or assembly defect in these mutants, though clearly this effect differs for the male X and must therefore also be linked to dosage-compensation mechanisms.

ISWI can play a role in global chromosome structure in *Xenopus* as well. Although ISWI is dispensable for the decondensation and replication of sperm chromatin (Demeret et al. 2002; MacCallum et al. 2002), remodeling of somatic nuclei in egg cytoplasm is critically dependent on ISWI (Kikyo et al. 2000). The remodeling of the somatic nuclei was measured by monitoring the energy-dependent loss of TBP (TATA-binding protein) from the incoming chromosomes. Although the normal *in vivo* function of this activity is not clear (it is clearly not analogous to sperm chromatin remodeling), it

has important implications for the mechanism of "epigenetic resetting" in somatic cell cloning and dedifferentiation (Wade and Kikyo 2002).

Role of ISWI in whole organisms

Unlike the specific functions of ISWI complexes discussed above, the role of ISWI in multicellular organisms as a whole can be summed up succinctly: it is essential. (In contrast, yeast cells lacking the *ISW1* and *ISWI2* genes are viable, raising the question of whether another complex substitutes for an essential function performed by ISWI in other species, or whether ISWI performs a function only essential to multicellular organisms.) Null mutations in *ISWI* or *NURF301* are lethal in the late larval/early pupal stage of development, and dominant-negative *ISWI* mutants result in loss of viability in each cell type they are expressed in (Deuring et al. 2000; Badenhorst et al. 2002). *Snf2h-/-* mice die during the peri-implantation stage, and *Snf2h* also appears critical for individual cell viability (Stopka and Skoultchi 2003). *Xenopus* also require ISWI for survival; ISWI-deficient *Xenopus* embryos die during late neurulation (Dirscherl and Krebs, unpublished data). In most of these experiments (with the exception of the NURF studies in *Drosophila*), the common subunit ISWI was targeted, so it remains to be seen which specific ISWI complexes are essential for cell viability and (or) development.

Chapter 3

Neural and eye-specific defects associated with loss of the Imitation Switch (ISWI) chromatin remodeler in *Xenopus laevis*

Introduction

The embryonic development of multicellular organisms is a complex and orderly process that depends on precise regulation of spatial and temporal patterns of gene expression. Cell specification and differentiation require that some gene loci become constitutively or inducibly expressed, while other loci become actively silenced. Cell type-specific patterns of expression and repression must often be maintained through subsequent rounds of cell division to preserve cell-lineage fidelity. Transcription activation and repression occurs in the context of chromatin, a complex of DNA and proteins that compacts DNA into the eukaryotic nucleus. The repeating structural unit of chromatin is the nucleosome, which is composed of 147 base pairs of DNA wrapped around an octamer of histone proteins. The compaction of DNA into chromatin, which functions to keep the genome organized within the boundaries of the cell nucleus, also suppresses gene activity. An essential step in gene activation includes the remodeling of nucleosomes at target promoters and enhancers, which facilitates binding of transcription factors. A combination of chromatin remodeling enzymes and histone modifying enzymes establish a steady level of transcriptional control in all eukaryotes (Khorasanizadeh 2004).

The ATP-dependent chromatin remodeling complexes use the energy of ATP hydrolysis to locally disrupt or alter the association of histones with DNA, or to slide

nucleosomes along the DNA. ATP-dependent chromatin remodeling complexes can facilitate gene activation or repression by helping transcription factors or histone modifying enzymes gain access to their targets in chromatin. All of the ATP-dependent chromatin remodeling complexes contain a catalytic ATPase subunit that belongs to the SWI2/SNF2 superfamily of proteins. These ATPases have been classified into several subfamilies, the largest of which are the SWI2/SNF2 group and the Imitation Switch (ISWI) group (Eisen et al. 1995).

Multiple ISWI-containing complexes have been identified in yeast, *Drosophila*, *Xenopus*, and mammals. ISWI complexes are involved in multiple nuclear functions, including transcriptional activation and repression, replication, and chromatin assembly (Dirscherl and Krebs 2004). Null and dominant negative *ISWI* mutants have demonstrated that ISWI is essential for cell viability and required for gene expression during development in *Drosophila* (Deuring et al. 2000), including expression of homeotic genes (Badenhorst 2002).

Homologs of *ISWI* have been identified in human and mouse (*Snf2h* and *Snf2l*) (Okabe et al. 1992; Aihara 1998; Lazzaro and Picketts 2001). Both *ISWI* homologs are expressed during development of the nervous system in mice, but they exhibit differential expression patterns (Lazzaro and Picketts 2001). *Snf2h* is transiently expressed in proliferating cell populations during embryogenesis and early postnatal development, while *Snf2l* expression is upregulated in terminally differentiated neurons after birth and persists in adult animals. A human complex containing SNF2L is involved in the induction of neurite outgrowth in tissue culture (Barak et al. 2003). Intriguingly, the *Snf2l*

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gene is localized to a region of the X chromosome associated with multiple X-linked mental retardation (XLMR) disorders (Lazzaro and Picketts 2001).

Four ISWI-containing complexes have been identified in *Xenopus* through biochemical fractionation of *Xenopus* oocytes (Guschin et al. 2000), three of which are homologs of ISWI complexes in other species. The most abundant ISWI complex in oocytes is xACF (ATP-dependent chromatin assembly factor). *Xenopus* also contains CHRAC (chromatin accessibility complex) and WICH (WSTF-ISWI chromatin remodeling complex) (Bozhenok et al. 2002). The subunit composition of the fourth ISWI complex has not been elucidated. Comparison of the remodeling activity of *Xenopus* ISWI complexes *in vitro* fails to reveal significant qualitative or quantitative differences (Guschin et al. 2000). The subunits unique to each ISWI complex could determine the specific nuclear function for each complex (chromatin assembly vs. transcriptional regulation vs. replication). Unquestionably, the developmental program of *Xenopus*, with an early period of rapid nuclear divisions, places a special burden on the machinery for chromatin assembly and remodeling. The multiple ISWI complexes described here may reflect those specialized demands.

A number of SWI2/SNF2-family ATP-dependent chromatin remodelers have been linked to developmental processes. There are several developmental disorders in humans associated with loss of function of chromatin remodelers. These include: ATRX mutations and mental retardation (Picketts et al. 1996) SMARCAL1 and Schimke immunoosseous dysplasia (Boerkoel et al. 2002), CSB and Cockayne syndrome (Citterio et al. 2000) and SNF2H and Williams syndrome (Bochar et al. 2000). Williams

syndrome is a developmental disorder linked to the ISWI-containing complex WICH, which contains the Williams Syndrome Transcription Factor (WSTF) and therefore suggests a possible transcriptional role for this complex. WICH also appears to have a direct role in the replication of heterochromatin (Bozhenok et al. 2002).

I wished to understand the functional diversity of ISWI complexes by defining the functions of different ISWI complexes in the whole organism. I began by analyzing the role of the one subunit common to all the ISWI complexes, ISWI itself. In this study I used *Xenopus laevis* to examine the expression pattern and regulatory effects of *ISWI* mRNA in the developing vertebrate embryo. *In situ* hybridization reveals *ISWI* mRNA to be localized almost exclusively in neural tissue. I inhibited ISWI function in early embryos by three methods: microinjection of antisense mRNA or microinjection of morpholino oligonucleotides to inhibit the translation of endogenous *ISWI* mRNA, and microinjection of mRNA encoding a dominant negative mutant of *ISWI.* I have shown that ISWI knockdowns result in gastrulation defects, incomplete closure of the neural tube, delayed development, misexpression of neural-specific genes, eye malformationsand the formation of cataracts. These results reveal an essential role for ISWI both in early development and in later stages of neural development.

Materials and Methods

RNA for Microinjection:

A 3500bp *ISWI* cDNA cloned into pBSKS was kindly provided by Dr. Paul Wade (Emory University). This *ISWI* clone was digested with *KpnI* and *PstI* and a 343bp fragment corresponding to nucleotides 2143-2486 was isolated and inserted into pBSSK to create a plasmid capable of transcribing an antisense RNA with the T3 promoter. Transcripts were synthesized using Megascript ™ (Ambion, Austin, TX). For in situ hybridization the same procedure was used except a digoxigenin-labeled UTP was incorporated. A plasmid capable of transcribing a full length *GFP* mRNA was provided by Dr. Richard Harland (UC Berkeley), and was used as an RNA injection control in these experiments. mRNA was transcribed using Megascript™ (Ambion, Austin, TX) and tailed using a Poly (A) Tailing Kit™ (Ambion, Austin, TX).

Morpholinos:

A 25-mer antisense morpholino (5'-GCTTTCCGCAGACATGACTCGCAGC-3') was designed against the 5' UTR of *Xenopus ISWI* immediately adjacent to the translation start (Gene Tools, LLC, Philomath, OR). Per Gene Tools recommendations, morpholinos were resuspended at 1mM and injected into embryos to give final concentrations in the range of 1-10 μM (10 μM H 80 ng total injected morpholino). A sample of morpholino was also lyophilized and resuspended at 2 mM high concentration injections. For a control, I use a standard control 25-mer (5'-CCTCTTACCTCAGTTA CAATTTAT-3') available from Gene Tools, LLC (Philomath, OR). This oligo has no target (except in reticulocytes from thallasemic humans with a specific α-globin

mutation) and is a commonly used negative control in antisense morpholino experiments. A 25-mer antisense morpholino (5'-GGCGTAGCCATCTAATGTTCTGGAG-3') was also designed against *xbrm* (*Xenopus brahma*).

Mutagenesis:

A dominant negative *ISWI* mutant of the Wade ISWI plasmid was created using the mutagenic primers 5'GGCTGATGAAATGGGTCTAGGAGCGACTTTGCAGACC-3' and 5'GGTCTGCAAAGTCGCTCCTAGACCCATTTCATCAGCC-3', converting the lysine at position 612 to an alanine. Mutagenesis was performed using Quickchange™ (Sigma, St. Louis, MO).

Microinjection:

Adult *Xenopus* were purchased from Xenopus Express (Plant City, FL). Embryos were obtained by in vitro fertilization and microinjection into the one cell stage was carried out by standard methods (Sive et al. 2000). Antisense RNA was injected at concentrations of 200, 400 and 1000 pg/nl. Morpholinos were injected at 500 μM, 1 mM and 2 mM (equivalent to 4, 8, 16 ng/nl), as suggested by the manufacturer. GFP was injected at 1000 pg/nl. 10 nl was injected into each embryo. Embryos were incubated at 16°C for 24-48 hours and staged according to Nieuwkoop and Faber (Nieuwkoop and Faber 1967). Extensive GFP staining was observed throughout embryos in which *GFP* mRNA was injected, indicating that diffusion of injected material from the injection site was not a problem.

Protein isolation and immunoblotting:

Protein was collected at stage 12/13 according to Merzdorf (Merzdorf and Goodenough 1997). A final concentration of 1μM PMSF was added to the protein isolation buffer, and DIFP, chymostatin and Trasylol were omitted from this buffer. Samples were then run on an SDS-page gel and transferred using standard methods (Sambrook and Gething 1989). Dr. Paul Wade generously provided ISWI antibody. E-Cadherin antibody (5D3) was obtained through the Developmental Studies Hybridoma Bank at the University of Iowa (Iowa City, IA).

Whole mount in situ hybridization:

Albino embryos were collected after *in vitro* fertilization and fixed in MEMFA according to Sive et. al (2000) (Sive et al. 2000). (Embryos were then rehydrated and assayed according to Sive et al. (2000) with the following changes: RNAse step was omitted, and a second preantibody incubation step with 2% BMB blocking reagent and 20% sheep serum in MAB for 1 hour at room temperature was added. AP buffer minus 2 mM levamisol was used as detection buffer. NBT/BCIP was used for the detection method. Negative controls for in situs were performed using ISWI sense strands; examples are shown in Appendix B.

Histology:

Fixed embryos were dehydrated though a graded series of ethanol and xylene, embedded in Paraplast Plus (Oxford Labware, St Louis, MO) and serial sectioned at a thickness of 7μm. Sections were collected on albumin-subbed slides (Mayer's fixative, (Humason 1972)). Sections were stained using Ehrlich's hematoxylin and counterstained

in Eosin following the protocols of Humason (1972). Coverslips were applied using Permount (Fisher Scientific, Pittsburgh, PA). Color images were captured using a Spot digital camera (Diagnostic Instruments, Inc. Sterling Heights, MI).

RT-PCR:

Total RNA was isolated using RNAWIZ™ (Ambion). One step real-time RTPCR was performed using Lightcycler™ reagents and the Cepheid Smartcycler (real time PCR). Reactions contained 1 μg total RNA and 2.5 pmol of primer, and were subjected to 25 cycles. RNA concentration range was confirmed with a 10 fold dilution series. Annealing temperatures were optimized for each primer set. Products were detected using Cyber Green during the real-time PCR reaction and/or running the final products on a 1-2% agarose gel. Data was quantified on 1-2% agarose gels using imaging software (Kodak 1D Image Analysis Software version 3.5). PCRs were performed a minimum of three times with samples from independent injection experiments. Primers were obtained through Sigma Genosys (St. Louis, MO). Primer sequences for *Hoxb9*, *MyoDb*, *EF1α*, and *NCAM* were obtained through Xenbase (www.xenbase.org). *Xmeis1b* was previously described (Maeda et al. 2001). Sequences for other primers are as follows:

BMP4: 5-'CCATGCCAGCCTCATACC-3' + 5'-GCTGGTCGGTCTCTCAGG-3'

Shh; 5'-GGTTCGACTGGGTCTATTACG-3' + 5'-CGATGAACATGAGGAAGTCG-3'

Slug: 5'-GGACTTAACTCCTGCAGG-3' + 5'-GGATCGTTGCTGGATTGTCTAGG-3'

Sox9: 5'-GGAGACTTCTGAATGAGGG-3' + 5'-GCTGGATATCTGTCTTGGG-3'

Pax6: 5'- CCGAGAAATGTCGCAGGG-3' + 5'- GGAATTACACAGTCCCTGGG-3'

Chromatin Immunoprecipitation:

Groups of 100 embryos were fixed in MEMFA (0.1 M Mops pH 7.5, 2 mM EGTA, 1 mM MgSO4, 3.7% formaldehyde) for 30 min, rinsed briefly and homogenized in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCL pH 8.1, 1 μl/ml Leupeptin, 1 mM PMSF, 1 μl/ml Pepstatin). Homogenized embryos were passed through a 20 gauge needle (20 passes). Chromatin was sonicated to yield DNA fragments between 200 and 500bp. Debris was removed by centrifugation for 10 min at 5,000 rpm at 4°C, supernatant was collected and spun again. DNA was quantified by measuring the absorption at 260 nm. Each sample was then diluted to 0.1 μg/μl in lysis buffer. Subsequent steps were performed as described (Strahl-Bolsinger et al. 1997; Kuo and Allis 1998). Input and immunoprecipitated material was detected via slot blot.

Results

Xenopus ISWI is expressed in neural tissues

Two nearly identical *ISWI* genes have been isolated from *Xenopus*, *ISWI 1* (Demeret et al. 2002) and *ISWI 2* (Guschin et al. 2000), which differ only by a 9 amino acid stretch that is not present in *ISWI 2* (amino acids 179-188 of *ISWI 1*). Probes and antibodies that detect both ISWIs reveal that ISWI is maternally deposited in the oocyte, and is also expressed continuously throughout embryonic development (Demeret et al. 2002), though localization of expression was not addressed in that study.

As a first step in determining the function of ISWI in *Xenopus*, I performed in situ hybridization in whole-mount embryos to determine the patterns of expression of ISWI in different developmental stages. The in situ probe recognizes both *ISWI 1* and *ISWI 2*. The pattern of ISWI staining in selected stages (stages 18 and 40) is shown in figure 1A-J (see also figure 2D-F). My results indicate that *xenopus ISWI* mRNA is localized to neural tissues throughout development. Specifically, *ISWI* is detected in the neural folds, in the cranial crest/brachial arches, in the otic vesicle, and to some extent in the migrating hypaxial muscles in early embryos. In later stages, *ISWI* is expressed throughout the brain and spinal cord. *ISWI* also exhibits strong eye staining in all stages. These results were surprisingly analogous to the recent findings that xBaf57 (a homolog of a subunit of mammalian and *Drosophila* SWI/SNF complexes) increases the expression of neural markers in ectoderm explants and is expressed in mesoderm during gastrulation and the nervous system during the neurula and tailbud stages of the *Xenopus* embryo

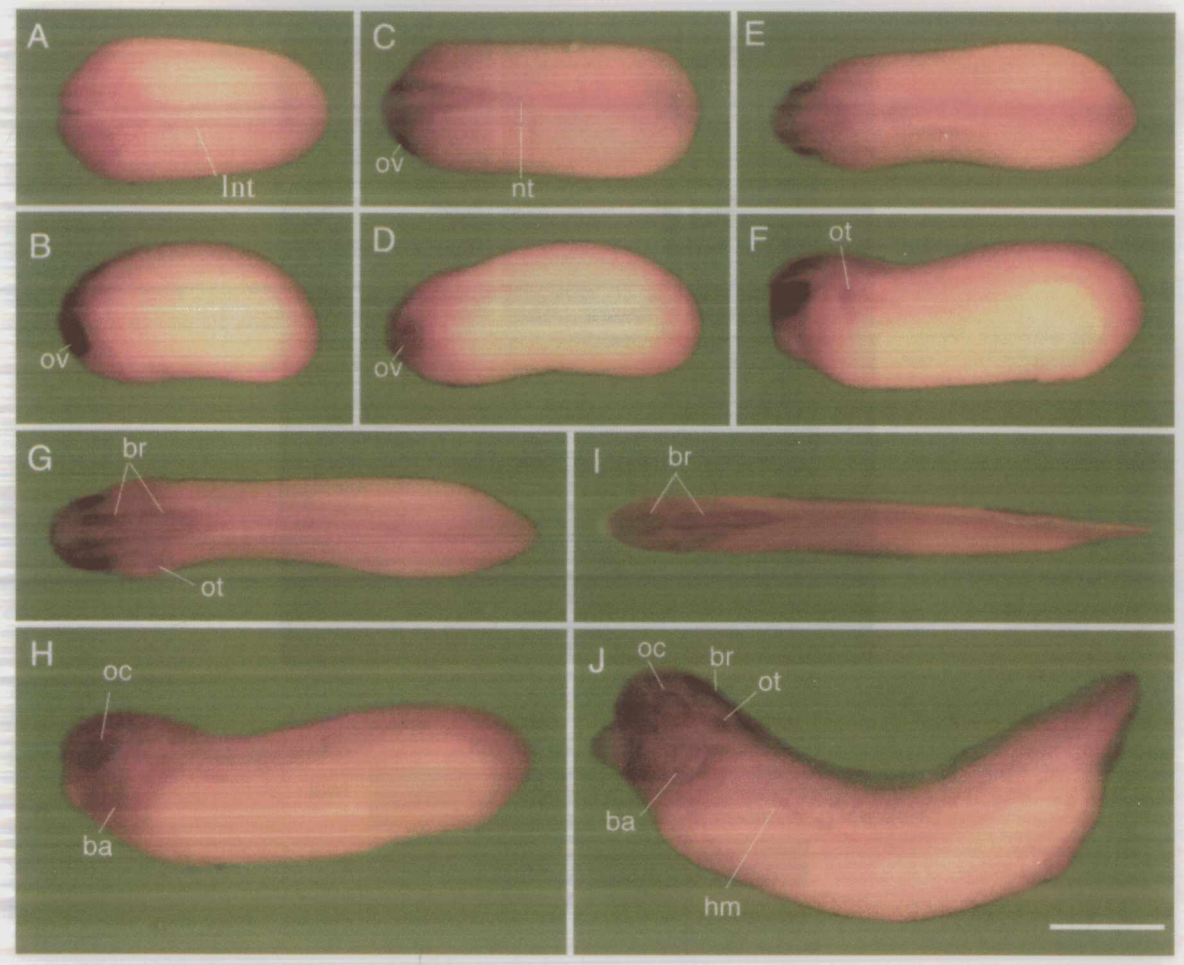

Figure 3.1 Whole mount in situ hybridization of *Xenopus* embryos with a 300bp *ISWI* digoxigenen-labeled probe. **A-J**: Corresponding dorsal and left lateral views of stage 18 (A-B), 24 (C-D), 26 (E-F), 28 (G-H) and 33 (I-J) embryos showing expression of ISWI in regions of the neural tube, brain, and optic vesicles. All embryos are oriented with the anterior end towards the left side of the figure. lnt: lateral neural tube; nt: neural tube; ov: optic vesicle; ot: otic vesicle; oc: optic cup; br: brain, ba: brachial arches; hm: hypaxial muscles. Scale bar equals 1 mm. For controls see appendix B.

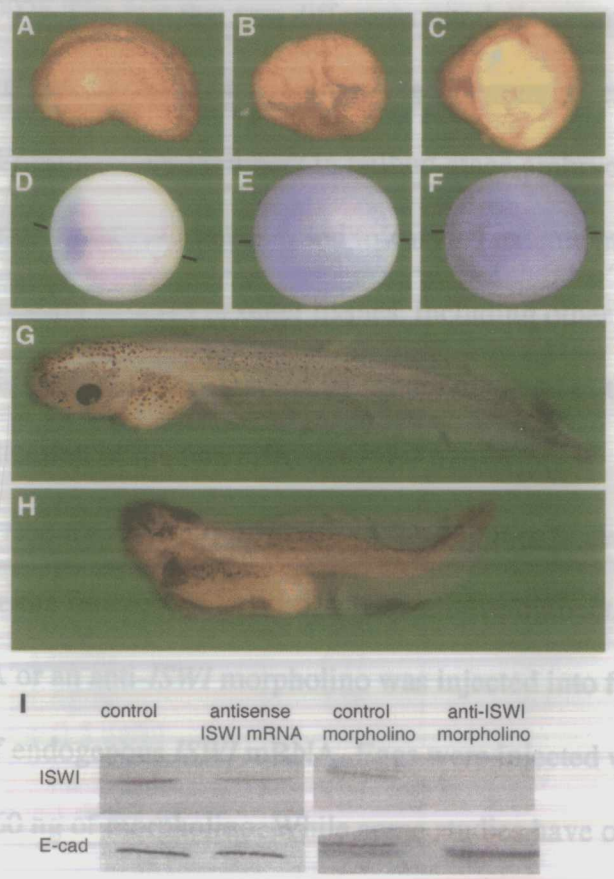

Figure 3.2 *Xenopus laevis* embryos injected with antisense *ISWI* RNA or anti-*ISWI* morpholino. **A-C:** Embryos photographed when control-injected embryos reached approximately stage 20-22. Embryos were injected with either 8 ng/nl control morpholino (A), 0.4 ng/nl of antisense ISWI RNA (B; representative of phenotype observed for injection of 0.2 and 1 ng/nl as well) or 8 ng/nl anti-*ISWI* morpholino (C; also representative of other of 4 ng/nl and 16 ng/nl morpholino inections). **D-F:** Whole mount in situ hybridization of stage 12 (D), 13 (E) and 14 (F) embryos with the *ISWI* probe described in Figure 1, showing early differential expression of ISWI. These are dorsal views with the anterior to the left side of the figure. Black dashes indicate the midline for each embryo. **G-H:** Embryos injected with 8 ng/nl of either control morpholino (G) or anti-*ISWI* morpholino (H). The embryo in H represents one of the very few embryos to survive the initial gastrulation defect at high morpholino concentrations. Scale bar: 1 mm. **I:** Western blot showing reduced levels of ISWI protein in embryos injected with 1 ng/nl of ISWI antisense RNA (left), or 8 ng/nl anti-ISWI moropholino (right), compared to control embryos injected with nanopure water or control morpholino (8 ng/nl), respectively. Antibody against E-cadherin (E-cad) is used as a loading control.

(Domingos et al. 2002). The fact that two different remodeling complexes are involved in nervous system development is very intriguing. A recent comprehensive survey of expression of SWI2/SNF2 family members in tailbud-stage embryos revealed that in fact all SWI2/SNF2 family members are expressed to varying extents in the brain, but exhibit greatly varying expression patterns in other tissues, including other neural tissues (Linder et al. 2004). Note that the in situs described by Linder et al and in this work do not distinguish the localization of the two different *ISWI* transcripts.

xISWI expression is required for normal gastrulation and neural development

To determine the function of ISWI in *Xenopus* development, either a 300bp antisense *ISWI* RNA or an anti-*ISWI* morpholino was injected into fertilized eggs to inhibit translation of endogenous *ISWI* mRNA. Eggs were injected with 2, 4 or 10 ng of RNA or 40, 80 or 160 ng of morpholino. While some studies have obtained results with lower concentrations of morpholinos, this concentration range was chosen according to the manufacturer's recommendations and is consistent with other published studies (Audic et al. 2001). For controls, eggs were injected with 10 nl of water, 10 ng of *GFP* mRNA, or 40, 80 or 160 ng of a control morpholino (see tables I and II). Embryos were allowed to develop at 16°C. Representative injected embryos are shown in figure 2A-C and G-H. At approximately 30 hours post-fertilization, when control embryos had reached stages 18-22 (figure 2A), an interesting phenotype began to emerge. Anti-*ISWI* injected embryos showed widespread failure of gastrulation and disruption of neural tube

Table 3.1 Neural phenotypes in injected embryos.

	Concentration (x 10 nl injected per embryo)	Total # of embryos	# of embryos with neural defects[a]	% of embryos with neural defects
Uninjected		1252	31	2%
Water		998	89	9%
GFP mRNA	1 ng/nl	1117	167	15%
Control MO	4 ng/nl (0.5 mM)	573	81	14%
	8 ng/nl (1 mM)	538	21	4%
	16 ng/nl (2 mM)	484	61	13%
Anti-*ISWI* mRNA	0.2 ng/nl	933	434	47%
	0.4 ng/nl	599	452	75%
	1 ng/nl	500	442	88%
Anti-*ISWI* MO	4 ng/nl (0.5 mM)	689	228	33%
	8 ng/nl (1 mM)	575	488	85%
	16 ng/nl (2 mM)	284	253	89%
DN-*ISWI* mRNA	1 ng/nl	631	507	80%
DN-*ISWI* mRNA +WT *ISWI* mRNA	1 ng/nl + 0.5 ng/nl	148	63	43%
DN-*ISWI* mRNA +WT *ISWI* mRNA	1 ng/nl + 1 ng/nl	229	77	33%

[a]Includes failure of gastrulation, neural tube closure, brain, eye and spinal deformities

Table 3.2 Cataract formation in injected embryos.

	Concentration (x 10 nl injected per embryo)	Total # of embryos	# of embryos with cataracts	% of embryos with cataracts
GFP mRNA	1 ng/nl	97	0	0
Control MO	8 ng/nl (1 mM)	273	0	0
Anti-*ISWI* MO	8 ng/nl (1 mM)	150	133	89%
DN-*ISWI* mRNA	1 ng/nl	109	83	76%

closure (figure 2B, antisense *ISWI* RNA-injected embryo; figure 2C, anti-*ISWI* morpholinoinjected embryo). At the highest doses of antisense or morpholino, close to 90% of the embryos exhibited these defects, compared to only 13-15% gastrulation failure in the equivalent control injections (table 1). Injection of 40 ng of the control morpholino does not affect development (e.g. figure 2G, embryo injected with control morpholino). Although it was already known that ISWI is present in the oocyte and throughout early development (Demeret et al. 2002), the early defects in gastrulation and neurulation led us to wonder whether *ISWI* is already exhibiting differential expression at these early stages. I therefore performed in situ hybridizations in embryos between stages 10 and 14. Representative in situs are shown in figure 2D-F. ISWI stains diffusely throughout the dorsal half of stage 10 embryos (not shown), but begins to localize to the anterior end by stage 12 (figure 2D), and shows clear neural plate and neural fold staining in stages 13/14 (figure 2E-F). This confirms that ISWI expression is rapidly localized to presumptive neural tissue early in development, consistent with the early critical role revealed in the antisense/morpholino injections.

A percentage of antisense- or morpholino-injected embryos survive the early defects and go on to reveal later developmental abnormalities. The example shown in figure 2E illustrates an anti-*ISWI* morpholino-injected embryo (40 ng) at approximately stage 40 (compare to the control morpholino-injected embryo in panel G). This embryo exhibits spinal deformities and dramatically reduced brain and eye development. At lower doses of ISWI inhibition (e.g. 40 ng of anti-*ISWI* morpholino), more embryos

survive the early gastrulation defects and go on to exhibit a spectrum of later neural defects, including severe eye defects, described below.

To confirm that ISWI translation was specifically inhibited in these embryos, I isolated total protein from stage 12 embryos that were injected with either 1 ng/nl antisense *ISWI* RNA or 8 ng/nl anti-*ISWI* morpholino. Total protein was also isolated from water or control-morpholino embryos at the same stage. The protein was run on an SDS-page gel and screened by western blot for ISWI protein, using an anti-ISWI antibody generously provided by Dr. Paul Wade (Emory University, Atlanta, GA). Processed ISWI protein runs at about 137 kDA. Western analysis reveals that ISWI protein levels are reduced but not eliminated in the antisense-injected embryos (figure 2F, left). In contrast, ISWI was completely undetectable in the morpholino-injected embryos (figure 2F, right). For loading controls, I used both Coomassie staining (not shown) and detection of E-cadherin protein, which was detected at similar levels in all samples. This clearly indicates that both the antisense *ISWI* RNA and the anti-*ISWI* morpholino are successfully reducing or preventing the translation of endogenous *ISWI* mRNA. The consistency between the phenotypes observed for both antisense RNA and morpholino methods strongly suggest the defects are due to the specific loss of ISWI (and not the result of non-specific toxicity of the anti-*ISWI* morpholino), and the western data supports this conclusion. A third method of ISWI inhibition using a dominant negative approach yields the same results (figure 4). In addition, I have also designed a morpholino that inhibits expression of *xbrm* (*Xenopus brahma*), a SWI2/SNF2-family member related to *ISWI*. This morpholino, injected at the same concentrations as the anti-

ISWI morpholino, does not result in gastrulation or neural defects but rather in later developmental defects unrelated to the defects observed in ISWI-deficient embryos (E. E. Brown, S.S.D. and J.E.K., unpublished results).

xISWI is required for normal expression of neural genes

Formation of the central nervous system (CNS) in vertebrates is initiated during gastrulation and depends on the inductive interaction of the ectoderm and the adjacent dorsal mesoderm. The CNS is characterized by overt anteroposterior (AP) and dorsoventral patterning (Doniach 1993; Mathis 2002). Nieuwkoop has described the predominant concept of how AP patterning is formed in a two-step model (Nieuwkoop 1955). The first step, "activation," is thought to specify anterior neuroectodermal structures, such as the forebrain. The second step is "transformation" where anterior neural tissue is respecified to more posterior fates such as midbrain, hindbrain and spinal cord. A number of genes have been identified whose regulation is critical in this process (Weinstein and Hemmati-Brivanlou 1999; Knecht and Bronner-Fraser 2002).Many ATP-dependent chromatin remodelers have been shown to act by regulating transcription, both positively and negatively. To determine whether ISWI controls expression of genes involved in neural development, I initiated a search for specific gene targets of ISWI. I tested whether expression of a number of known neural marker genes was affected in the injected embryos. Total RNA was purified from the antisense- or morpholino-injected embryos and subjected to RT-PCR using primers against a variety of neural markers expressed at different times and positions in neural development. These include *BMP4*

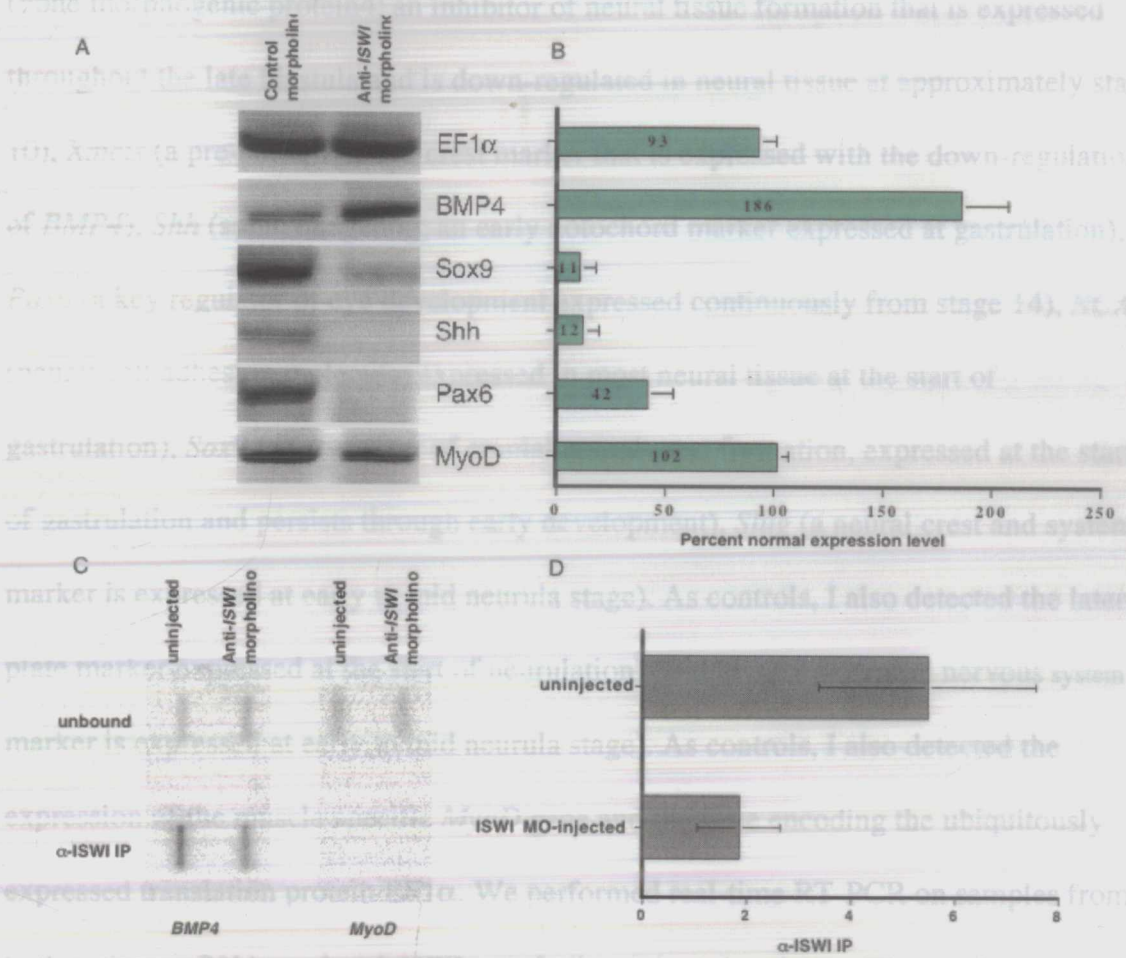

Figure 3.3 RT-PCR of tissue-specific genes in anti-*ISWI* morpholino-injected embryos. Total RNA was collected from embryos at the specified stages and real-time RT-PCR was performed with specific neural gene primers. **A.** Products of RT-PCR reactions. *EF1α* was tested at stages 10, 12, 13, 15, 18 and 20. The stage 13 data is shown and is representative of all stages tested. Other genes shown were analyzed at the following stages: *BMP4*, stage 10/11; *Sox9* and *Shh*, stage 13; *Pax6* and *MyoD*, stage 15/16; *HoxB9*, stage 18/20. **B.** Quantitative data from real-time RT-PCR, showing the level of expression (as percentages) of each gene compared to the levels in control-injected embryos. Each bar represents the average from a minimum of three injection experiments. Standard errors are shown. **C.** Representative slot blots of chromatin immunoprecipitations with anti-ISWI antibody. Chromatin was extracted from stage 12/13 embryos; comparable results were obtained with later stages. Left panel was probed for *BMP4*, right panel was probed for *MYOD*. **D.** Average enrichment of ISWI at *BMP4* in uninjected and anti-ISWI MO-injected embryos (stage 12/13). Standard errors are shown.

(bone morphogenic protein4; an inhibitor of neural tissue formation that is expressed throughout the late blastula and is down-regulated in neural tissue at approximately stage 10), *Xmeis* (a pre-pattern neural crest marker that is expressed with the down-regulation of *BMP4*), *Shh* (sonic hedgehog; an early notochord marker expressed at gastrulation), *Pax6* (a key regulator of eye development expressed continuously from stage 14), *NCAM* (neural cell adhesion molecule; expressed in most neural tissue at the start of gastrulation), *Sox9* (a progenitor of cranial neural crest formation, expressed at the start of gastrulation and persists through early development), *Slug* (a neural crest and system marker is expressed at early to mid neurula stage). As controls, I also detected the lateral plate marker expressed at the start of neurulation), and *HoxB9* (a central nervous system marker is expressed at early to mid neurula stage). As controls, I also detected the expression of the muscle specific *MyoD* gene and the gene encoding the ubiquitously expressed translation protein EF1α. We performed real-time RT-PCR on samples from both antisense RNA- and anti-*ISWI* morpholino-injected embryos. The results were the same for both; data for the morpholino injections are shown in figure 3. Figure 3A shows representative PCR products from the RT-PCR reactions. The average level of expression for each gene in the *ISWI* knockdown embryos is expressed as a percentage of the level of expression in control-injected embryos (figure 3B). Each bar represents the data from a minimum of three separate injection experiments, and standard errors are shown. *Xmeis, Shh, Pax6, NCAM, Sox9, Slug* and *HoxB9* all show a decrease in expression compared to embryos injected with water, GFP or control morpholino (figure 3 and data not shown), suggesting that ISWI is acting at a very early step in this developmental cascade, which is

consistent with the gastrulation defect I observe. The muscle-specific *MyoD* was unaffected by the presence or absence of ISWI, supporting the hypothesis that the primary role of ISWI is in the development of neural tissue.

ISWI binds directly to the* BMP4 *promoter

Consistent with the down-regulation of neural genes, expression of *BMP4* was *increased* approximately 2-fold in anti-*ISWI* morpholino-injected embryos (figure 3). Reduced BMP activity has been shown to induce anterior-neural tissue from non-neural ectoderm (Harland 1997; Harland and Gerhart 1997). *BMP4* is normally down-regulated in neural tissue in order for neurulation to begin. Since ISWI has been implicated in both activation and repression of genes, I wished to test whether ISWI is directly targeted to the *BMP4* promoter, where it could act as a repressor in neural tissues. I therefore used chromatin immunoprecipitation (ChIP) analysis to detect ISWI protein at *BMP4*. I used the anti-ISWI antibody to immunoprecipitate cross-linked, sheared chromatin, and measured the enrichment of *BMP4* or control sequences (*MYOD*) using slot blots. Representative blots are shown in figure 3C, and the average enrichment of ISWI at *BMP4* is shown in Figure 3D. ISWI protein is detected at *BMP4* in vivo (figure 3C, left) consistent with a direct role for ISWI in *BMP4* regulation. In contrast, no ISWI is detected at the *MyoD* gene, the expression of which is unaffected by the presence or absence of ISWI (figure 3A-B). Furthermore, the *BMP4* signal is lost in anti-ISWI morpholino-injected embryos. These results strongly suggest that ISWI acts as a repressor of *BMP4* in neural tissue. This analysis was performed in whole embryos;

ChIPs of neural vs. non-neural tissue will help confirm whether ISWI is a repressor of *BMP4* in neural tissue or an activator of *BMP4* in other tissues.

A Dominant-Negative ISWI mutant also inhibits development

Antisense interference using direct injection of antisense RNA or morpholinos is a powerful method for functional inhibition of a target gene in all tissues in early development. However, it is more difficult to target injected antisense/morpholinos to specific tissues. I have developed a dominant-negative mutant of *xISWI*, *DN-ISWI*, which can ultimately be placed under the control of tissue-specific or inducible promoters in transgenic frogs. I identified the invariant lysine in the ISWI ATPase domain (K612) and mutated it to an alanine (figure 4A). The mutation was confirmed through sequencing and the plasmid was named ISWI-K612A. Mutation of this conserved lysine has been used to create catalytically inactive mutants of yeast *SWI2* (Khavari 1993; Richmond and Peterson 1996) and human *Brg1* and *hbrm* (de la Serna et al. 2001) These ATPase_ mutants assemble into remodeling complexes, competing with the wildtype ATPase for complex assembly. To confirm that the ISWI-K612A mutant behaves as a dominant negative in *Xenopus* embryos, I tested whether the presence of the mutant *ISWI* in early embryos could recapitulate the phenotype I observed in the antisense/morpholino experiments. A 3500bp *DN-ISWI* transcript was produced using the T7 promoter and a poly-A tail was added for better translation efficiency. A *GFP* transcript was also

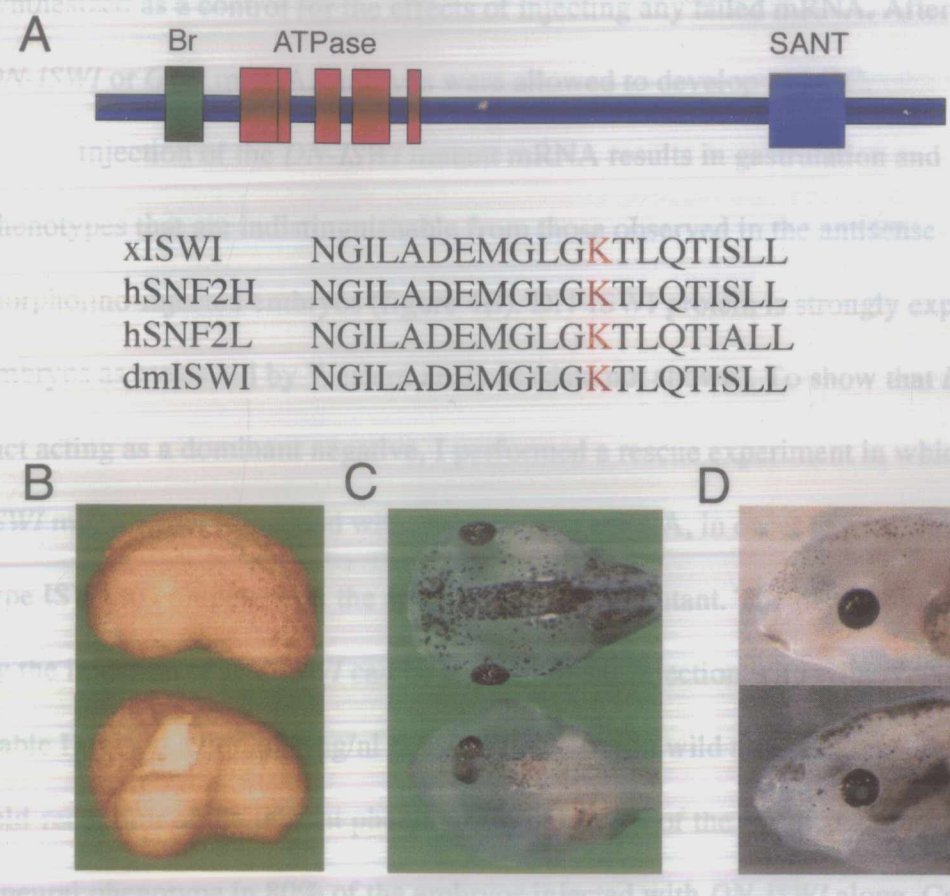

Figure 3.4 An ATPase mutant of ISWI acts as a dominant negative in vivo. **A.** The conserved lysine in the *Xenopus* ISWI ATPase domain, which is essential for ISWI's catalytic activity, was located and mutated to an alanine. An alignment of *Xenopus*, human, and *Drosophila* ISWI homologs shows the relevant region, with the invariant lysine (K) shown in red. **B.** Injection of 1 ng/nl *DN-ISWI* mRNA recapitulates the gastrulation defect observed in antisense- or anti-*ISWI* morpholino-injected embryos (bottom panel). Top panel shows a control embryo injected with 1 ng/nl *GFP* mRNA. **C.** Failure of brain development in *DN-ISWI* mRNA-injected embryo (bottom); compare to stage 45 embryo from same egg clutch injected with *GFP* mRNA (top). **D.** Cataract development in stage 43 embryo injected with *DN-ISWI* mRNA (bottom; gray spot in center of eye); control *GFP* mRNA-injected embryo is shown above.

synthesized as a control for the effects of injecting any tailed mRNA. After injection of *DN-ISWI* or *GFP* mRNA, embryos were allowed to develop at 16°C.

Injection of the *DN-ISWI* mutant mRNA results in gastrulation and neural fold phenotypes that are indistinguishable from those observed in the antisense- and morpholino-injected embryos (figure 4B). DN-ISWI protein is strongly expressed in embryos as measured by Western analysis (data not shown). To show that *DN-ISWI* is in fact acting as a dominant negative, I performed a rescue experiment in which wild-type *ISWI* mRNA was coinjected with the *DN-ISWI* mRNA, in order to provide more wild type ISWI to compete with the dominant negative mutant. The neural phenotype caused by the injection of *DN-ISWI* can be rescued by co-injection with wild type *ISWI* mRNA (table I). Coinjection of 1 ng/nl *DN-ISWI* + 0.5 ng/nl wild type *ISWI* resulted in a two fold-reduction of the mutant phenotype to only 43% of the injected embryos, compared to a neural phenotype in 80% of the embryos injected with *DN-ISWI* alone. Coinjection of 1 ng/nl of both *DN-ISWI* and wild type *ISWI* reduces the neural phenotype to only 33% of the injected embryos. It is also important to note that the fact that DN-ISWI causes the same phenotypes as inhibition of ISWI suggests that it is the chromatin remodeling/ATPase activity per se of ISWI that is critical for its function in development.

As I observed with antisense RNA and morpholino injections, injection of lower concentrations of DN-ISWI mRNA permitted a number of embryos to survive the early gastrulation defects, allowing us to detect phenotypes at later stages. Comparable to the morpholino-injected embryos, DN-ISWI mRNA-injected embryos exhibit a spectrum of defects in brain and eye development. These include extreme developmental delays in

eye development: for example, at stages 35-38, ISWI-deficient embryos had small, flattened lenses still attached to the overlying ectoderm (separation normally occurs at stage 33), resembling lens placodes as normally seen at stage 25-26. Another dramatic phenotype is shown in Figure 4C. Some embryos injected with *DN-ISWI* mRNA exhibit a catastrophic failure of forebrain development, coupled with malformed eyes that are mislocalized to the central axis of the animal, essentially occupying the space created by the reduction of forebrain tissue.

ISWI-deficient embryos develop posterior subcapsular cataracts and have defects in retinal differentiation

Late stage survivors of *DN-ISWI* mRNA injections develop a striking phenotype at approximately stage 38/40: the development of congenital cataracts in the vast majority of the survivors (figure 4D; table II). This phenotype also occurs in anti-*ISWI* morpholino-injected embryos; in some batches of injected embryos every survivor eventually developed clouded lenses, while the cataract phenotype never appeared in control injections (table II). In addition, these embryos appear to be blind, in that they are unresponsive to shadows or objects moving above them, stimuli that cause control embryos to swim rapidly away from the objects, substantiating the extent of these lens and retinal defects. The cataractous embryos do, however, respond to currents or touch, consistent with specific visual impairment.

There are many different types of congenital cataracts, such as those identified in humans (Amaya et al. 2003). We therefore initiated a collaboration with Dr. Jonathan Henry (University of Illinois Urbana) to perform histological analyses to further

characterize the specific eye defects in the ISWI-deficient embryos. Sections from both control and anti-ISWI morpholino-injected embryos at stages 38 and 45 are shown in figure 5A-F. The cataractous lenses exhibit abnormal cell proliferation on the posterior surface, leading to the accumulation of large densely stained, basophilic cells (figure 6E, asterisk) resulting in a form of posterior subcapsular cataract and a severe lenticonus condition. Liquefied, apoptotic cells also appear to be present.

In general the ISWI morpholino knockdown eyes appear to be retarded in their development when compared to control eyes. Anti-ISWI morpholino-injected embryos exhibit defects in retinal differentiation, and cell adhesion (compare figure 5A,C to D,F). younger stage 38 eye cups appear to have many loose rounded cells. Some cells in the younger embryos also appear to be picnotic, highly condensed, fragmented and apoptotic (figure 5D). Later defects are also apparent. For instance, cells of the ganglion layer do not appear to be arranged in a single layer (figure 5E-F). These cells are also closely associated with the lens, which is normally well separated at this time (compare figure 5B and E). There is no clear distinction of the inner and outer nuclear layers nor the formation of a thin outer plexiform layer (compare figure 5C and F).

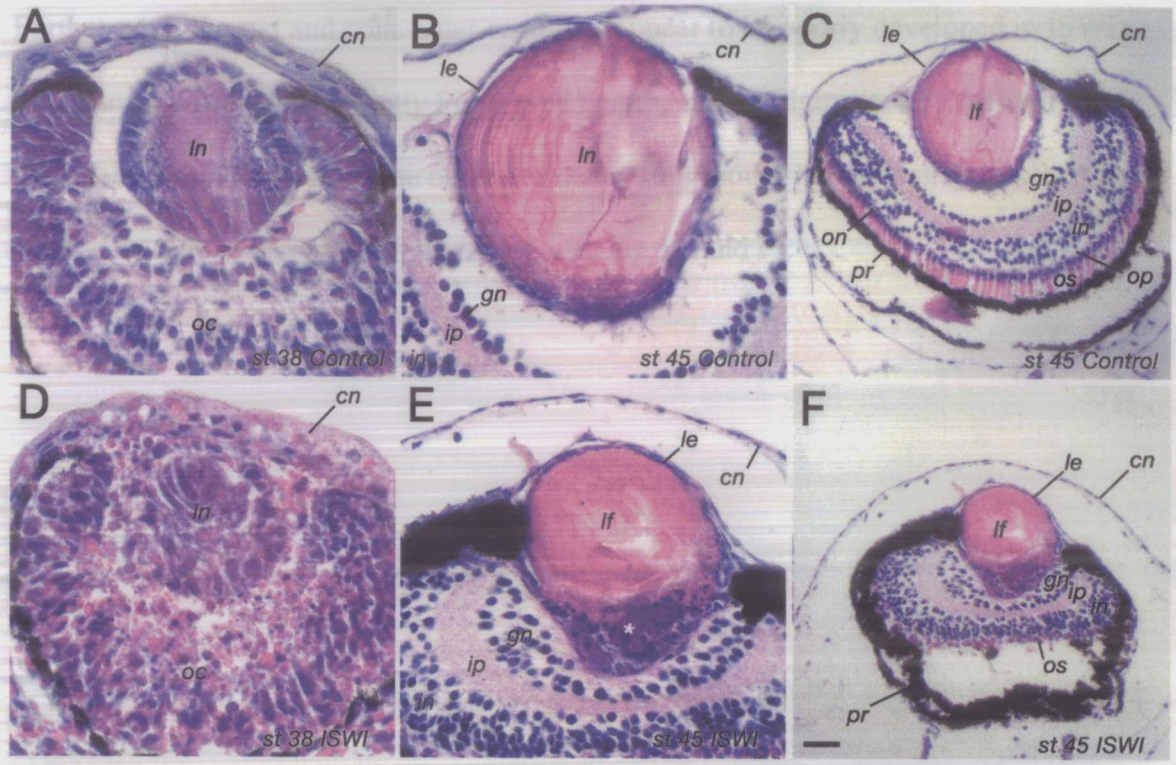

Figure 3.5 Cross sections through normal control and defective eyes in ISWI morpholino knockdown embryos. **A.** Normal stage 38 eye showing optic cup, lens and cornea. Note presence of large central, primary lens fiber cell "nucleus" and additional secondary lens fiber cells at the periphery of the lens. **B-C.** Corresponding high and low magnification views of the normal eye in a stage 45 animal. Note large mass of enucleated lens fiber cells and thin nucleated lens epithelium. Also note normal arrangement of differentiated cell layers within the eye cup. **D.** Stage 38 ISWI morpholino knockdown eye. Note that the lens is more poorly developed compared to (A) and contains a much smaller mass of primary lens fiber cells. Other lens and retinal cells appear to be more highly rounded. **E-F.** Corresponding high and low magnification views of the stage 45 ISWI morpholino knockdown eye. Note presence of large basophilic, nucleated cell mass (cataract) on the posterior surface of the lens (asterisk). Defects are also apparent in the development of the retinal layers (see text for further details). Cn, cornea; gn, ganglion layer; in, inner nuclear layer; ip, inner plexiform layer; le, lens epithelium; lf lens fiber cells; ln, lens; oc, optic cup; on, outer nuclear layer; op, outer plexiform layer; os, rod and cone outer segments; pr, pigmented retinal epithelium. Scale bar equals 25 μm for A-B and D-E, 50 μm for C and F.

Furthermore, the rod and cone outer segments appear to be poorly developed in ISWI knockdown animals. Frequently I observed that the retinal pigmented epithelium is detached from the rod and cone outer segments, suggesting that there may be other changes in cell adhesion, as well (compare figure 5C and F).

Discussion

Imitation Switch and Xenopus laevis

I have shown that *Xenopus ISWI* mRNA is located specifically in neural tissue, including brain, neural fold, hypaxial muscle, the optic vesicle and cup, auditory vesicles and spinal cord. Early inhibition of ISWI protein production causes a lethal phenotype of incomplete gastrulation and neurulation. The mRNA levels of the neural-specific genes *Xmeis, shh, slug, sox9, Pax6*, and *Hoxb9* are greatly reduced in embryos injected with antisense *ISWI* RNA or anti-*ISWI* morpholino, while the muscle-specific gene *MyoD* is unaffected. However, the mRNA of *BMP4*, a gene normally down-regulated in neural tissue as neurulation begins, is over-expressed in ISWI-deficient embryos. I propose that ISWI may normally facilitate the formation of neural tissue by repressing genes that inhibit neural tissue such as *BMP4*, which is supported by the fact that ISWI binds directly to the *BMP4* gene in vivo, and this interaction is perturbed upon injection of anti-*ISWI* morpholino. This model does not exclude an additional role for ISWI in activating genes that specify neural tissue development. It is possible that ISWI has multiple neural-specific gene targets and is continuously required throughout development.

The knockdown of ISWI also results in the formation of congenital cataracts. ISWI is not normally expressed in the lens ectoderm. Hence, I surmise that these cataracts are due to the retarded and abnormal development of the retinal tissues in these embryos, leading to abnormal induction of the lens, and possibly to the inadequate production of key growth factors required to sustain normal lens development and differentiation. These lens defects could also stem from abnormal planar induction of the

placodal ectoderm during the early phase of lens induction (Henry and Grainger 1990) These effects may be directly or indirectly related to the abnormal regulation of *BMP4* expression in the neural ectoderm, or to other key genes involved in controlling and patterning eye development such as *Pax6* or *shh.* (Crossley et al. 2001; Zuber et al. 2003). For instance, it is known that BMP4 plays a key role in eye development and lens induction (Furuta and Hogan 1998). It is also known that BMP4 regulates cell proliferation and apoptosis in the brain and optic cup (Trousse et al. 2001).

I have created a functional dominant-negative *ISWI* mutant, which will be an excellent tool to dissect ISWI function, by interfering with ISWI function at specific times or in specific tissues during development. By placing DN-ISWI under control of neural-specific promoters, for example, I can test the role of ISWI in specific neural pathways.

This study has focused on the role of ISWI, which is involved in at least four different complexes in *Xenopus.* Ultimately, we hope to determine which of these ISWI complexes are responsible for the specific defects I observed by targeting subunits unique to each complex, using the methods described in this work. These future experiments will allow us to examine the specific interactions of chromatin remodelers and their targets during neural development in *Xenopus.*

Chromatin remodeling and neural development

Chromatin remodeling complexes are often thought of as ubiquitous factors that control the expression of large numbers of genes. It is therefore somewhat surprising to find that so many remodeling complexes appear to be specific for neural tissue, including

Xenopus, human, murine and *Drosophila* ISWI homologs. In addition to the ISWI complexes, other SWI2/SNF2 family members appear to be critical in neural development. The human CHD5 remodeler is localized in neural tissue and is suspected to play a role in embryonic development (Thompson et al. 2003). BAF53b, part of the human SWI/SNF remodeling complex, is localized in postmitotic neurons and may create neuronal-specific patterns of chromatin accessibility (Olave et al. 2002). BAF57, a *Xenopus* SWI/SNF subunit, works in conjunction with Xsmad7 to increase expression of neural markers in *Xenopus* ectodermal explants (Domingos et al. 2002). Numerous SWI2/SNF2 family members exhibit neural expression patterns in *Xenopus* (Linder et al. 2004). Finally, the human SNF2L complex hNURF is enriched in the brain and regulates human *Engrailed*, a homeodomain protein that regulates neuronal development in the mid-hindbrain (Barak et al. 2003).

ISWI is turning out to be a surprisingly diverse subfamily of the SNF2 superfamily. *Drosophila* has three ISWI-containing complexes, yeast and *Xenopus* have four, and mammals appear to have seven distinct ISWI complexes. ISWI-containing complexes tend to have fewer subunits (2-4) relative to SNF2 subfamily containing complexes (~12). In biochemical assays, SNF2-subfamily complexes tend to disrupt nucleosome structure, while ISWI-subfamily complexes have been observed to enhance chromatin structure by equalizing nucleosome spacing, and to assemble nucleosomes (Lusser and Kadonaga 2003). ISWI also has a unique requirement for histone tails for nucleosome remodeling (Clapier et al. 2002). Recently the crystal structure of the nucleosome recognition module of ISWI was determined and an ISWI-specific DNA

binding domain called SLIDE, in a region essential for H4 binding, was identified adjacent to the SANT domain (Grune et al. 2003). It is clear from many studies how important ISWI-containing complexes are to the development and/or function of each organism in which these complexes have been described. Each chromatin-remodeling complex, despite the similarities of their biochemical activities *in vitro*, may have far more specialized roles in gene expression and other cellular functions than previously suspected.

Why so many ISWI complexes?

The ISWI family is unique among the SWI2/SNF2 superfamily for the sheer diversity of different complexes. ISWI complexes are generally small (2-5 subunits) compared to other SWI2/SNF2 complexes such as SWI/SNF, RSC, or NuRD, which have on the order of 8-15 subunits. (The only large ISWI complex, in fact, is the ISWI-cohesin complex, which gets most of its bulk from NuRD.) Why pack every subunit you could ever need into a single massive SWI/SNF complex, while spreading ISWI out into 3-7 different complexes, many of which barely deserve the name "complex?" (Two-subunit complexes are sometimes called heterodimers).

There seem to be two key differences between the ISWI family and most of the other ATP-dependent remodelers: the diversity of functions performed and the differences in targeting. Most of the other ATP-dependent remodelers that have been characterized seem to function primarily in transcription, either activation or repression. Different remodelers regulate different sets of genes, which leads to different biological outcomes, but the basic function remains the same. The vast numbers of subunits are

responsible for targeting of the complexes to all the sites of action, and for interacting with other factors needed for the transcriptional control, such as histone modifying complexes and basal transcription machinery.

For ISWI complexes, transcriptional regulation represents only a subset of the roles that must be satisfied. Many of the roles performed by ISWI complexes don't require site-specific targeting at all: replication and chromatin assembly occur everywhere, roles in transcriptional elongation may apply to many or all genes, cohesin loading is likely to have only minor sequence specificity at most, and global changes in chromosome structure are not likely to be mediated site-directed targeting of ISWI. ISWI shows up in pericentric heterochromatin, telomeric chromatin, and the nucleolus. These may all represent examples of targeting to structures rather than sequences. Of course, some ISWI complexes are likely to have site-specific roles, particularly those that are involved in transcriptional regulation of particular genes, such as NURF.

In a sense, ISWI is an all-purpose tool that can be tossed into many different toolboxes. All ISWI containing complexes share the ability to slide nucleosomes, a clear biological function of ISWI in vitro (Langst and Becker 2001a; Langst and Becker 2001b; Kang et al. 2002; Kassabov 2002 Nov; Flaus and Owen-Hughes 2003; Kukimoto et al. 2004) that has also been detected in vivo (Fazzio and Tsukiyama 2003). ISWI can be fitted with different handles, such as a subunit to interact with the replication machinery or a subunit or two to recognize an elongating RNA polymerase. We have seen ISWI appear in a complex with cohesin and NuRD; we may find that other

remodelers or histone modifiers occasionally have a use for ISWI as well. The count is up to 19, how many more places will we find ISWI in the future?

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Appendix A

In situ hybridization controls

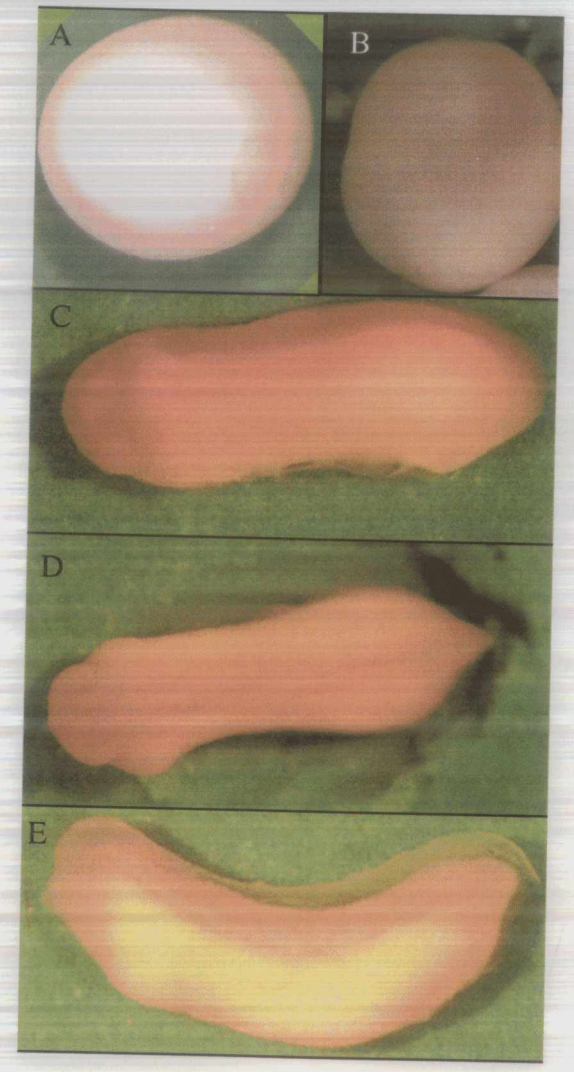

Figure B.1 Representative negative controls for in situ hybridization. This group represents embryos incubated with ISWI sense strand digoxygenin labeled probe. A-B: stage 10/12, C: stage 25/26, E: approximately stage 30.